Effect of peracetic acid

at low concentrations

on fish health and water quality

Dissertation

zur Erlangung des akademischen Grades

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This thesis is dedicated to my parents (Haishi Liu and Xiangfen Ju) and my wife (Haijie Li). Thank you for the endless love and support.

Contents:

Zusammenfassung

Peressigsäure (PES) hat seit kurzem als Desinfektionsmittel in der Aquakultur Einzug gehalten. Gegenüber anderen konventionellen Desinfektionsmitteln besitzt es in niedrigsten Konzentrationen (ca. 1 mg l^{-1}) eine hohe Effektivität. Des Weiteren hat die Anwendung von PES kaum einen negativen Einfluss auf die Umwelt. Die Applikation von PES in Aquakulturanlagen erfolgt direkt über das umgebende Haltungswasser. Dies geht mit einem direkten Kontakt der Mikroorganismen und der Fische mit dem Wirkstoff einher. Aus diesem Grund ist generell ein Einfluss auf die Fischgesundheit und die Wasserqualität zu erwarten. Dieser hypothetische Einfluss ist bislang jedoch unzureichend untersucht worden. In der Praxis werden zumeist zwei Applikationsstrategien verfolgt: 1. Wiederholende Kurzzeitpulsapplikationen mit relative hohen PES-Konzentrationen (1-2 mg l^{-1}) und 2. Die kontinuierliche Applikation mit relative geringen PES-Konzentrationen (≤ 0,2 mg l^{-1}) in der Wasserzufuhr. Die potentiellen Unterschiede dieser zwei Strategien speziell auf die Fischgesundheit und die Wasserqualität sind bislang unklar.

In der vorliegenden Studie wurden Effekte einer PES-Applikation auf die Fischgesundheit und die mikrobielle Aktivität in identischen Durchflussystemen untersucht. Diese Systeme garantieren eine optimale Wasserqualität. Regenbogenforellen wurde als Testorganismen gewählt. Verschiedene Stressparameter, Parameter des oxidativen Stresses, Wachstum, Kiemenhistologie und Parameter der angeborenen Immunantwort wurden zur Bewertung der Fischgesundheit herangezogen. Sauerstoff, pH und die visuelle Biofilmformation wurden kontinuierlich kontrolliert um die mikrobielle Aktivität zu

interpretieren. Dazu wurde zweimal wöchentlich mit 1 mg l^{-1} PES (Pulsbehandlungen) im Haltungswasser und kontinuierlich mit 0,2 mg l^{-1} PES am Zulauf exponiert und verglichen.

Die Ergebnisse belegen, dass die Pulsapplikationen mit 1 mg l^{-1} PES, im Gegensatz zur kontinuierlichen Applikation mit 0,2 mg l^{-1} PES die Fische stressten. Die Fische adaptierten sich jedoch an die PES-Pulsapplikationen. Dies wurde durch nachfolgend weniger heftige Reaktionen der Fische post applicationem, reduzierte Kortisolausschüttungen und unveränderte Reaktionen auf andere Stressoren deutlich. Obwohl die PES-Applikation leichte Hyperplasien in den Kiemen induzierte, war kein Einfluss auf das Wachstum und die angeborene Immunantwort feststellbar. Dies kann als ein Beleg für den fehlenden Einfluss der PES-Exposition auf die Fischgesundheit bewertet werden. PES induzierte unabhängig von den Applikationsstrategien oxidativen Stress in den Fischen. Als Antwort auf die PES-Applikation steigerten die Fische ihre antioxidative Antwort gegen die freien Sauerstoffradikale speziell in den Kiemen und im Serum. Unabhängig von den extrem geringen PES-Konzentrationen in der kontinuierlichen Applikation wiesen die Fische einen geringen oxidativen Stress auf. Der oxidative Stress der Fische in der Pulsexposition war hingegen periodisch nachweisbar. Daraus ist zu schlussfolgern, dass die Fische zwischen den Pulsexpositionen, wenn keine PES vorhanden war, Phasen der Erholung hatten. Das Fehlen dieser Erholungsphase in der kontinuierlichen Expositionsgruppe führte zu einer signifikanten Reduktion der Antiprotease-Aktivität im Serum. Dies impliziert das Risiko einer chronischen Entzündung.

Die antimikrobiellen Effekte sind stark von der PES-Konzentration abhängig. Die Pulsapplikation mit 1 mg l^{-1} PES hemmte die mikrobielle Aktivität stärker als die

kontinuierliche Exposition durch einen stärkeren oxidativen Stress. Dadurch wurde der Biofilm fast vollständig erodiert, und die mikrobielle Sauerstoffsverbrauch und nitrifikation inhibiert. Die PES-Konzentrationen in der kontinuierlichen Exposition waren zu gering um signifikante Effekte auf den Mikroorganismen auszuüben. Des Weiteren kann das PES-Zerfallsprodukte, die Essigsäure und Acetate, eine potentielle Kohlenstoffquelle für die Mikrobiota darstellen. Der daraus resultierende stärkere Biofilm kann durch die Besiedelung mit fakultativen Fischpathogenen eine Gefahr für die Fischgesundheit darstellen. Auf Grund des starken antimikrobiellen Effekts und des geringen Risikos die Fischgesundheit zu beeinträchtigen, werden periodisch regelmäßige PES-Applikationen in Konzentrationen von 1-2 mg l^{-1} empfohlen.

Effekte einer PES-Applikation auf Spiegelkarpfen und die Wasserqualität in stark belastetem Wasser einer geschlossenen Aquakulturkreislaufanlage (RAS) wurde ebenso untersucht. Die Induktion einer schlechten Wasserqualität erfolge durch den Stopp der Wasserzufuhr zu den Tanks. Simultan zu den Wasserstopps erfolgte eine Applikation mit 1 mg l^{-1} PES. Die Stressantwort, Kiemenhistologie und die angeborene Immunantwort wurde mit nicht mit PES exponierten Kontrollfischen verglichen. Der Stopp der Wasserzufuhr steigerte die gesamte heterotrophen Bakteriendichte (GHBD) auf das Sechsfache. Im Gegensatz dazu wurde in den Expositionsgruppen die GHBD um 90% gesenkt. Der stark mikrobiozide Effekt der PES-Exposition verbesserte die Gesundheit der Kiemen, verhinderte bakterielle Infektionen welche in den Kontrollgruppen kurzzeitig festgestellt wurden.

Zusammenfassend erhält PES appliziert periodisch in Konzentrationen von 1-2 mg l^{-1}, im Fall der optimalen Wasserqualität, die gute Wasserqualität mit geringfügiger

Beeinträchtigung der Fischgesundheit. In der Aquakulturproduktion, in welcher die Wasserqualität meistens durch die hoher Besatzdichte und organischer Belastung verschlechtert wird, verhindern regelmäßige prophylaktische PES-Applikationen Infektionen und begünstigen die Fischgesundheit.

Abstract

Peracetic acid (PAA) has been recently introduced to aquaculture as a sustainable disinfectant. It has great advantages over conventional disinfectants by having high effectiveness and low environmental impact at very low concentrations (around 1 mg L^{-1}). The application of PAA in aquaculture facilities is realized by adding PAA products to the rearing water. This leads to unavoidable exposure of fish and microorganisms (surface-attached and waterborne) to PAA. Consequently, a potential impact of PAA on fish health and microbial activities is expected. This potential impact, however, has been poorly studied. In aquaculture practice, two strategies are broadly used to apply PAA: short term high dose (1-2 mg L^{-1} PAA) periodic/pulse applications or continuous low dose (≤ 0.2 mg L^{-1} PAA) application related to the makeup water flow. The potential difference between these two strategies remains unclear, especially concerning their impacts on fish health and water quality.

In the present study, the impact of PAA on fish health and microbial activities was tested in identical flow-through systems controlled with optimal water quality. Rainbow trout was selected as the model fish. Various parameters of stress, oxidative stress, growth, gill histology and innate cellular/humoral immunity were measured to indicate fish health. Oxygen, pH and visible biofilm formation were continuously monitored to interpret changes of microbial activities. In addition, the application strategies, biweekly pulse applications of 1 mg L^{-1} PAA in the rearing water and the continuous application of 0.2 mg L^{-1} PAA in the inflow, were compared.

The results indicate that pulse applications of 1 mg L^{-1} PAA stressed the naïve fish during the first exposure, while the continuous application not. Fish could progressively adapt to PAA-induced stress, as indicated by less intensive behavioral reaction, reduced cortisol release and unaffected response to another stressor. Although the exposure to PAA induced slight hyperplasia in fish gill, the growth and innate immunity were affected, indicating unaffected overall health. PAA induced oxidative stress in fish, regardless of the application strategies. In response, fish enhanced their antioxidative defense, especially in gill and serum, to scavenge excessive free radicals induced by exposure to PAA. Despite of extremely low PAA concentration measured during the continuous application, the constant input of PAA induced a constant mild oxidative stress to fish. In contrast, the oxidative stress induced by pulse 1 mg L^{-1} PAA applications was periodic present. Consequently, fish had periodic recovery phases when the pulse PAA applications were absent. The lack of recovery phases in fish exposed to the continuous PAA application resulted in a significant reduction of antiprotease activity in serum. This implies a potential risk of chronic inflammation.

The antimicrobial effect of PAA depended on applied concentration. The pulse applications of 1 mg L^{-1} PAA strongly inhibit microbial activities by inducing a strong oxidative stress. As a result, the biofilm in fish tanks was nearly erased, and the microbial oxygen consumption and nitrification were inhibited. In contrast, the PAA concentration during the continuous application was so low that only a minor antimicrobial effect was observed. In addition, the degradation products, acetic acid and acetate, were beneficial for the biofilm formation by providing organic carbon. The enhanced biofilm may become a potential risk by providing protective shed for opportunistic pathogens. Due to the strong antimicrobial

effect and low risk to affect fish health, it's recommended to apply PAA periodic at high concentrations (1-2 mg L^{-1}) with sufficient intervals.

The impact of PAA on fish health and water quality was also tested in a mirror carp recirculating aquaculture system (RAS) challenged with bad water quality. The challenge of bad water quality was realized by transient water stops in fish tanks. Simultaneous to the transient water stops, PAA at 1 mg L^{-1} was applied. The stress, gill histology and innate cellular immunity were compared in fish with or without simultaneous PAA treatments. The transient water stops caused a 6-fold increase of heterotrophic bacterial density in water, while the simultaneous PAA treatments caused a 90% decrease of heterotrophic bacterial density. The strong antibacterial effect of PAA significantly improved the gill health of fish, and effectively prevented bacterial infections, which were short-term present in fish exposed to transient water stops alone.

To sum up, PAA applied periodically at 1-2 mg L^{-1} in optimal water quality is effective to maintain the water quality at a low cost of scarifying fish health. In production-scale aquaculture facilities, where the water quality is often deteriorated by high stocking density and organic load, regular applications of PAA are especially beneficial to enhance fish health and prevent potential infections.

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List of Abbreviations

PAA	Peracetic acid
RAS	Recirculating aquaculture system
OPO	Ozone produced oxidants
UV	Ultraviolett
LFUS	Low frequency ultrasound
NOEC	No observed effect concentration
FFA	Free fatty acid
CFU	Colony forming units
TAN	Total ammonia nitrogen
PMA	Phorbol 12-myristate 13-acetate
DMSO	Dimethyl sulfoxide
NBT	Nitroblue tetrazolium
MTT	Thiazolyl blue tetrazolium bromide
OD	Optical density
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
TAC	Total antioxidant capacity
DCF	2’, 7’-dichlorodihydrofluorescein
FCR	Feed conversion ratio
AP	Alkaline phosphatase
TSP	Total serum protein
MP	Myeloperoxidase
SO	Serum osmolality
SF	Stimulation factor
EG	Eosinophilic granulocytes
HP	Hyperplasia
PBS	Phosphate buffered saline
DPD	N, N-diethyl-p-phenylendiamine sulfate salt

List of publications

- **Published:**

1. Liu, D., Steinberg, C.E.W., Straus, D.L., Pedersen, L.-F., Meinelt, T., 2014. **Salinity, dissolved organic carbon and water hardness affect peracetic acid (PAA) degradation in aqueous solutions**. Aquacultural Engineering 60, 35-40.
2. Liu, D., Straus, D.L., Pedersen, L.-F., Meinelt, T., 2015. **Comparison of the Toxicity of Wofasteril Peracetic Acid Formulations E400, E250, and Lspez to Daphnia magna, with Emphasis on the Effect of Hydrogen Peroxide**. North American Journal of Aquaculture 77, 128-135.
3. Liu, D., Behrens, S., Pedersen, L.-F., Straus, D.L., Meinelt, T., 2016. **Peracetic acid is a suitable disinfectant for recirculating fish-microalgae integrated multi-trophic aquaculture systems**. Aquaculture Reports 4, 136-142.

- **Under Review:**

1. Liu, Dibo, Straus, David L., Pedersen, Lars-Flemming, Kloas, Werner, Meinelt, Thomas, 2016. **Fish-friendly prophylaxis/disinfection in aquaculture -Peracetic acid at low concentration is an adaptable stressor for the mirror carp after repeated applications** Aquaculture.

2. Liu, Dibo, Straus, David L., Pedersen, Lars-Flemming, Meinelt, Thomas. **Pulse versus continuous peracetic acid applications: effects on rainbow trout performance and water quality.** Aquaculture Engineering.

- **In process:**

1. Liu, Dibo, Straus, David L., Pedersen, Lars-Flemming, Meinelt, Thomas. **Regular applications of peracetic acid at low concentrations improve gill health and prevent bacterial infections in mirror carp in RAS.**
2. Liu, Dibo, Straus, David L., Pedersen, Lars-Flemming, Lazado, Carlo Cabacang, Meinelt, Thomas. **Response of rainbow trout to exogenous free radicals: effect on histology, innate immunity and inflammatory markers**.

List of academic presentations

1. Topic: Toxizität von Peressigsäure – eine Fallstudie an *Daphnia magna*.

   XV. Gemeinschaftstagung der European Association of Fish Pathologists (EAFP)

   Starnberg, Germany, 08-11. October 2014

2. Topic: Fish-friendly prophylaxis/disinfection: low concentration of peracetic acid is stress-free to mirror carp (*Cyprinus carpio*) after regular applications.

   17th International Conference on Disease Fish and Shellfish of EAFP

   Las Palmas de Gran Canaria, Spain, 7-11. September 2015

3. Topic: Pulse vs. Continuous: which is better for applying peracetic acid in RAS?

   11th International Conference on Recirculating Aquaculture and 2016 Aquaculture innovation workshop

   Roanoke, VA, USA, 19-21 August 2016

1. Introduction

1.1. Chemical characters and applications of peracetic acid

Peracetic acid (PAA) is a strong oxidizing agent. Its chemical formation is CH_3CO_3H. Because pure PAA is highly corrosive and flammable, it is normally diluted in water. PAA in water undergoes the following reaction (Wagner et al., 2002):

$$CH_3COOOH + H_2O \leftrightarrow CH_3COOH + H_2O_2$$

The reaction, namely hydrolysis, is reversible, so the PAA solution is the mixture of PAA, acetic acid, hydrogen peroxide (H_2O_2) and water. Commercial PAA solutions consist of stabilizers in addition to maintain the equilibrium of all components. The known stabilizers are normally phosphonates (Yuan et al., 1997a) and dipicolinic acid (Quick and Harrison, 2010). Commercial PAA solutions are normally applied by diluting in water to reach a certain PAA concentration. In this case, the stabilizers are diluted and the original equilibrium is broken. Beside of hydrolysis, PAA might degrade in forms of spontaneous decomposition and transmission metal-catalyzed degradation (Yuan et al., 1997b). Both reactions are as follows, respectively:

$$2\ CH_3COOOH \rightarrow 2\ CH_3COOH + O_2$$

$$CH_3COOOH \xrightarrow{transmission\ metal} CH_3COOH + O_2 + other\ products$$

As a result of the degradation, the residues of PAA are biodegradable and harmless. For this reason, PAA has been introduced as a sustainable alternative to sewage treatment (Kitis, 2004), bleaching industry (Yuan et al., 1997b), fruit/vegetable disinfection (Alvaro et al., 2009), hospital disinfection (Loukili et al., 2006) and ballast water treatment (de Lafontaine et al., 2009). Most recently, PAA has been used as a sustainable disinfectant in aquaculture,

preliminarily in recirculating aquaculture system (RAS).

1.2. Peracetic acid versus other disinfectants/disinfection procedures in RAS

Ozone was the first effective strategy for the disinfection in RAS. However, ozone is lethal to fishes at concentration of 0.01 mg O_3 L^{-1} (Summerfelt and Hochheimer, 1997). Although ozone degrades within seconds in the water, its degradation results in ozone produced oxidants (OPO). Chronic exposure to OPO above 0.06 mg L^{-1} can cause irreversible gill damage (Reiser et al., 2011). Ultraviolet (UV) irradiation is effective to kill waterborne bacteria in RAS. However, its effectiveness is restricted by the turbidity (Gullian et al., 2012) and particulate matters (Sharrer et al., 2005). To solve this problem, UV was combined with ozone to achieve stronger bacterial reduction effect (Sharrer and Summerfelt, 2007). Recently, low frequency ultrasound (LFUS) was introduced to disinfection in RAS (Bazyar Lakeh et al., 2013). The authors found that LFUS was effective to inactive parasites, such as ciliates, nematodes and crustaceans. Besides, it could improve the disinfection effectiveness of UV irradiation by breaking particulate matters and releasing embedded bacteria. However, LFUS is acutely harmful to fish at high intensity (Cobo et al., 2014). The potential chronic harm of LFUS to fish remains unknown. Therefore, LFUS for disinfection purpose is suggested to be bypassed from fish culture units. Similar to UV, it cannot disinfect surface-attached pathogens. Hydrogen peroxide (H_2O_2) is a sustainable disinfectant, because it releases hydroxyl radicals and degrades to water. The application of H_2O_2 in RAS is restricted by the relatively high effective dose and potential harm to the biofilter. The dose recommended for disinfection is ≥ 15 mg H_2O_2 L^{-1}, but the safe concentration for the biofilter

is < 5 H_2O_2 mg L^{-1} (Pedersen and Pedersen, 2012). In contrast, PAA was proven to be effective to kill most pathogens *in vitro* at a much lower concentration of around 1 mg L^{-1} (Pedersen et al., 2013); and this concentration was proved to have no effect on total ammonia nitrogen removal of the biofilter in RAS (Pedersen et al., 2009). Therefore, peracetic acid has great advantages over the other disinfectants/disinfection procedures.

1.3. Effectiveness of peracetic acid against fish pathogens

PAA has been proven to be effective against various fish pathogens *in vitro* (Smail et al., 2004; Meinelt et al., 2009; Straus and Meinelt, 2009; Marchand et al., 2012; Picon-Camacho et al., 2012; Straus et al., 2012; Meinelt et al., 2015). As summarized by Pedersen et al. (2013), the effective concentration of PAA to control common fish pathogens is around 1 mg L^{-1}. In addition, the effectiveness of PAA has been also proven in *in vivo* tests (Sudová et al., 2010; Jussila et al., 2011; Straus et al., 2012; Farmer et al., 2013). In some cases, however, the effectiveness of PAA was restricted, because PAA mainly disinfect the surface of fish (skin and gill) and has no effect on embedded pathogens. Despite of that, all fish pathogens have certain stages in their life cycles outside of the host fishes, which are vulnerable to PAA treatment. Therefore, PAA *in vivo* can still control the expansion of pathogens and prevent new infections.

1.4. Toxicity of peracetic acid to non-target aquatic organisms

As summarized by Wessels and Ingmer (2013), the toxicity of PAA is based on its unspecific oxidation potential, which can induce oxidative stress to all living cells. The

application of PAA in aquaculture leads to simultaneous exposure of PAA to fish. To avoid potential harms, the toxicity of PAA to fish has been investigated. Marchand et al. (2013) found that the 24-h-LC_{50} value of PAA in zebrafish (*Danio rerio*) embryos was 2.24 - 7.14 mg L^{-1} depending on water hardness. Straus et al. (2012) determined that the 24-h no observed effect concentration (NOEC) of PAA was 2.2 mg L^{-1} for yolk-sac fry and 1.3 mg L^{-1} for swim-up fry of channel catfish (*Ictalurus punctatus*). Higher concentrations caused degeneration of gill epithelium and mortality. These toxicity studies provided a guideline for the safe dose of PAA in aquaculture.

Beside of fish, the toxicity of PAA was also studied on non-target aquatic organisms. Liu et al. (2015) compared the toxicity of three commercial PAA products with various PAA : H_2O_2 proportions to *Daphnia magna*. The authors found that H_2O_2, as a component in PAA products, was similar toxic to *Daphnia* as PAA. Consequently, the total peroxide (PAA+ H_2O_2) concentration instead of PAA concentration alone determines the toxicity. As discussed by Wessels and Ingmer (2013), different organisms show various susceptibility against PAA. Among the microorganisms, the eukaryotic microalgae seem to be most tolerant to PAA. A representative species, *Tetraselmis chuii*, was found to be unaffected by daily PAA disinfections up to 2 mg L^{-1} by showing unaffected growth and photosynthesis (Liu et al., 2016b). This finding provides a possibility of fish-microalgae integrated RAS that can be routinely disinfected with PAA.

1.5. Degradation and application of PAA in aquaculture

In aqueous solutions, the degradation of PAA follows generally a first-order exponential

decay (Pedersen et al., 2009). Salinity, hardness and dissolved organic carbon were found to increase the decay variously (Liu et al., 2014). Since different aquaculture systems have various water parameters, the degradation of PAA is likely to differ. In RAS, the complete decay of PAA could be achieved within several hours post application (Pedersen et al., 2009; Pedersen et al., 2013). A faster complete decay within 30 min was observed in a raceway-RAS with high organic load and insufficient solids removal (unpublished case study in Hohen Wangelin, Germany). In this case, short exposure duration might lead to ineffective disinfection. Therefore, specific strategies for the application of PAA based on degradation under different water conditions are highly recommended.

Because of the high water retention rate in RAS, the nitrifying bacteria in the biofilter are simultaneously exposed to PAA during disinfection. Exposure to PAA at concentrations of less than 1 mg L^{-1} had no effect on the performance of the biofilter (Pedersen et al., 2009). If the applied concentration is higher than 1 mg L^{-1}, the authors suggested that the biofilter should be temporarily by-passed until the degradation lowered the concentration.

Another concern of applying PAA is the equal distribution of PAA in aquaculture facilities with different hydraulic characters. In tank-based facilities, an equal distribution of PAA in each tank can be realized by applying sufficient aeration in each tank. In raceway-based facilities, an equal distribution of PAA is difficult to achieve (Pedersen and Pedersen, 2012). Unequal distribution of PAA resulted in overdose of PAA in some areas and underdose in the others. Consequently, the effectiveness of disinfection was reduced, and the fish and nitrifying bacteria exposed to overdosed PAA might be harmed. A multi-spot and slower application helped to improve the equal distribution of PAA. Therefore, a good

understanding of the hydraulics in an aquaculture facility is the precondition for the application of PAA.

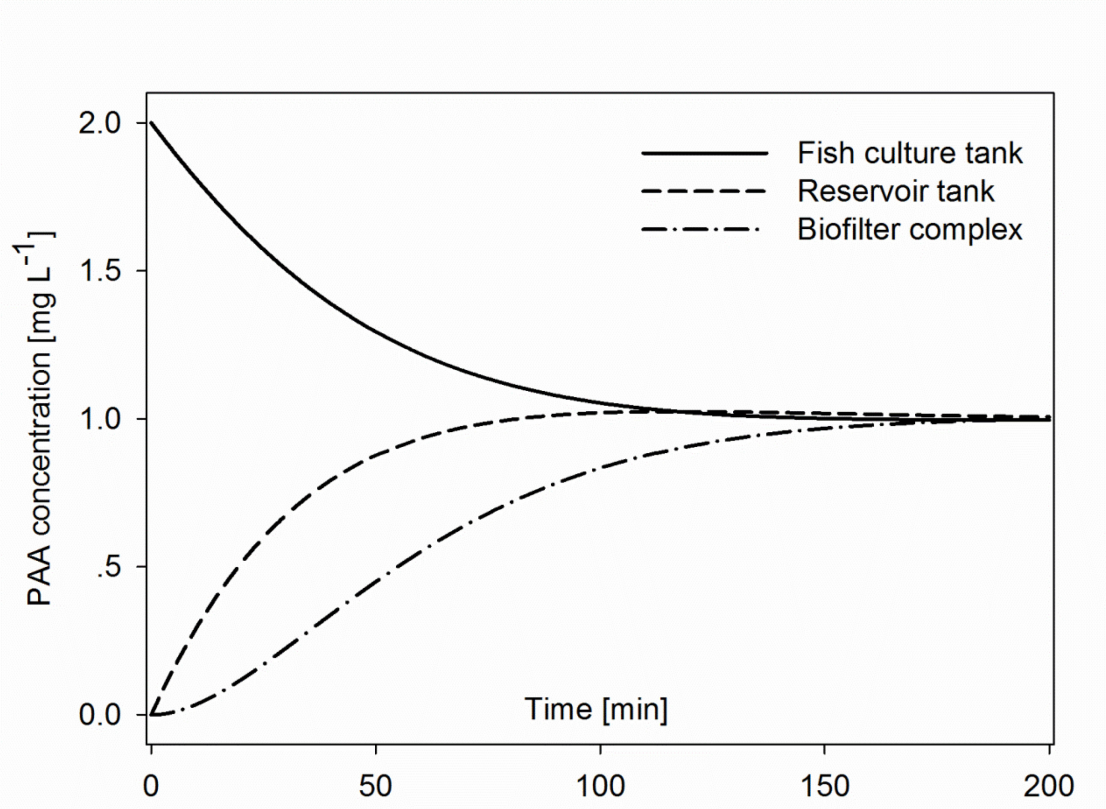

Figure 1 Theoretical PAA distribution model ignoring PAA decay in a pilot-scale RAS consisting of a fish culture tank (1000 L), a reservoir tank (600 L) and a biofilter complex (400 L). The Flow rate is 10 L min^{-1} (Liu et al., 2016b).

The hydraulics in RAS is, briefly, separated units connected by the water flow. As demonstrated in a pilot-scale RAS (Figure 1) established by Liu et al. (2016a), if PAA is added to the rearing unit at a given amount to achieve 1 mg L^{-1} in the entire RAS, PAA was only present in the rearing unit at a higher concentration (2 mg L^{-1}) before it progressively distributed to the rest parts of RAS. The distribution rate depends on the flow rate and the size of each unit. This finding supports a new strategy to avoid harm on the biofilter by the

PAA application in RAS: PAA should only be applied in the rearing unit. The applied concentration should be calculated based on the size of the rearing unit instead of the whole RAS. A reduction of the flow rate will slow down the distribution of PAA and decrease the final PAA concentration reaching the biofilter.

1.6. Remaining questions and objectives of the present study

Many management practices in aquaculture can induce fish stress, and corresponding actions to minimize stress have been investigated and implemented (Zahl et al., 2012). Although disinfection with PAA products decreases the potential risk of pathogenic diseases, the exposure of the disinfectants itself may also induce stress to fish (Powell et al., 2015). According to toxicology studies shown above, PAA at an effective concentration (1 mg L^{-1}) should be non-lethal to fish. Despite of that, it has been observed that zebrafish showed escaping behavior directly after the application of PAA (personal observation of Dr. Thomas Meinelt). This behavior clearly indicates that PAA might stress fish during disinfection. Moreover, multiple/repeated disinfections with PAA are necessary to maintain the long-term hygiene in aquaculture facilities. Pulse or continuous PAA application strategies can lead to repeated high concentration exposure or prolonged low concentration exposure, respectively, resulting in two scenarios. One scenario is that the fish may suffer from chronic stress, exemplified by chronic or constantly elevated plasma cortisol. As a result of increased compensatory metabolic activity, the fish lose their homeostasis resulting in poor growth and suppressed immunity (Bonga, 1997; Harris and Bird, 2000; Magnadóttir, 2006). Another scenario is that the fish may adapt to repeated or prolonged PAA applications and show

unaffected growth and immunity (Wilkie et al., 1996; Smith et al., 2011). Since different PAA concentrations and exposure durations are used in these two disinfection strategies, their impacts on fish health, especially stress condition, growth and immunity are likely to differ. As an oxidizing agent, PAA may also induce oxidative stress in fish (Elia et al., 2006). Because of different concentrations and exposure durations, the two disinfection strategies may also induce different oxidative stress in fish, and fish may show different strategies to compensate the PAA-induced oxidative stress. Similarly, disinfection strategies are potentially also affecting bacterial abundance and activity (Pedersen et al., 2010), however, not studied for trade PAA products. Biofilms are ubiquitous in aquaculture. They passively colonize surfaces (tank walls, bottom), and consist of mainly periphytic algae and bacteria (van Dam et al., 2002). Especially in RAS, biofilms are the fundamental of nitrifying biofilters (Malone and Pfeiffer, 2006). Biofilms affect various water quality parameters, such as ammonia and nitrite that are oxidized by nitrifying bacteria (Hagopian and Riley, 1998; Rurangwa and Verdegem, 2015); and hence affect water quality, such as oxygen and pH (Moriarty, 1997). Therefore, two disinfection strategies of PAA may result in different water qualities.

The aim of the present study is 1) to compare the potential different impacts of the mentioned application strategies of PAA on fish health, microorganisms and water quality in flow-through systems with optimal water quality; 2) to test the obtained results in a production-scale RAS challenged with bad water quality. The hypothesis arose from the personal observation of Dr. Thomas Meinelt, too. He observed that zebrafish showed less extensive escaping behavior along repeated PAA disinfections without compromise of

survival rate and reproduction success. Therefore, it was hypothesized that in optimal water quality, naïve fish may be stressed by first PAA applications. Subsequently, fish could progressively adapt to PAA treatment and show reduced stress, unaffected growth and immunity. Moreover, the pulse and continuous strategies are likely to induce different oxidative stress and antioxidative responses in fish. Likewise, they may affect microbial activities differently and result in different water qualities. In case that fish are challenged with bad water quality, it was hypothesized that the application of PAA may enhance the fish health by improving water quality.

2. Materials and methods

2.1. Fish and rearing systems

2.1.1. Rainbow trout in flow-through systems

Rainbow trout (*Oncorhynchus mykiss*) weighing 115 ± 10 g and of mixed sex were purchased from BioMar Research Centre (Hirtshals, Denmark) and were acclimated to the experimental system for 3 weeks. Prior to the experiment, fish were anesthetized with KALMAGIN 20% (Laboratory Centrovet, Santiago, Chile), individually weighed and equally distributed into 9 experimental tanks (18 fish per tank); mean stocking density (mean ± standard deviation) was 11.83 ± 0.14 kg m^{-3}. Subsequently, fish were acclimated for another week before treatments began.

The 180-L Plexiglas cylindrical flow-through experimental tanks (Figure 2; as depicted in Dalsgaard and Pedersen (2011)) were in a Guelph System. The tanks were further modified with in-tank aeration via an external airpump with tubing extending to a depth of 30 cm to an airstone, and an individual water pump (EHEIM Compact 1000, Deizisau, Germany) installed in each tank to create an identical radial flow downwards. A fixed inlet flow of 20 L h-1 to each tank originated from a common oxygen supersaturated water reservoir.

Feed (EFICO Enviro 920 4.5 mm pellets, BioMar) was offered daily at quantities equivalent to a feeding rate of 0.8% biomass to each tank from 17:30 to 17:40 via individual custom-made feeding automat (DTU-Aqua, Hirtshals, Denmark). Daily feed was progressively increased based on estimated growth using an expected feed conversion ratio of 1.0. Uneaten pellets and sediments were removed daily from the drain directly after feeding and the following morning. Temperature and dissolved oxygen concentration were measured

daily immediately after feeding with a Handy Polaris portable DO meter (OxyGuard®, Farum, Denmark). In case of low oxygen in the experimental tanks, the oxygen concentration in the common water reservoir was increased.

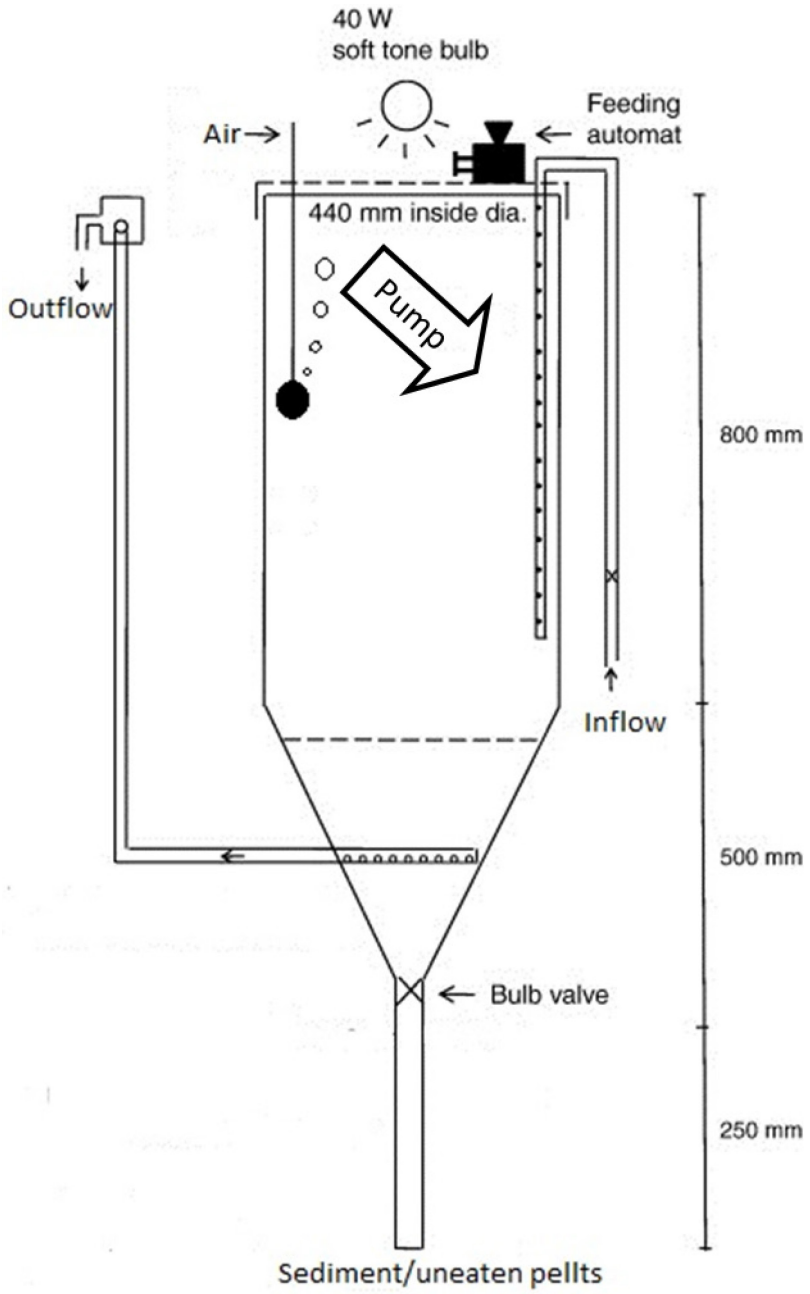

Figure 2 Modifications of the experimental tanks based on the design of Dalsgaard and Pedersen (2011)

2.1.2. Mirror carp in a RAS

Mirror carp (*Cyprinus carpio*) weighing 649 ± 183 g of mixed sex were purchased from a local fish farm and quarantined for 3 months until moved to a previously disinfected RAS. The rearing unit of the RAS consisted of 16 rectangular tanks with water volume of 300 L and continuous aeration. Each tank was stocked with 6 carp. The carp were acclimated for 6 months and fed daily with commercial pellets of about 1% biomass until the treatments began. The water temperature was 21-22 °C, the pH was 7.3-7.7 and the oxygen concentration was 8-8.5 mg L^{-1}.

2.2. Treatment and sampling

2.2.1. Rainbow trout in flow-through systems

The nine flow-through tanks were equally and randomly divided into 3 treatment groups. One treatment group received a pulse application of 1 mg L^{-1} PAA every Monday and Thursday. The dose was administered by pipetting (Eppendorf, Hamburg, Germany) 1.053 mL of the PAA product, Aqua Oxides (S. Sørensen, Thisted, Denmark), in 15 mL distilled water and subsequently adding this dilution slowly (10-15 sec.) into the respective tanks. The second treatment group received continuous application of a 1:500 dilution of Aqua Oxides at the rate of 0.195 mL min^{-1}; this was equivalent to dosing with 0.2 mg L^{-1} PAA in the inflow. The continuous treatment was administered with an ISMATEC® BVP Standard peristaltic pump equipped with PharMed® Ismaprene tubing (Cole-Parmer, Wertheim, Germany). The control group, without PAA exposure, received a sham treatment by adding 15 ml distilled water similar to the pulse treatment.

A series of water samples were taken from each tank during every second pulse treatment (Mondays) in the first 4 weeks. For each sampling period, water was collected immediately before, and 1, 2, 4, 8, 24, 28, 32 and 48 h after the pulse treatment. Water samples were collected from the tank outflow in 1-L glass bottles (SCHOTT, Mainz, Germany). On a non-pulse treatment day in the 5th week, fish in all tanks were additionally stressed by being gently harassed with a dipnet for 90 s. Water samples were collected immediately before, and 1, 2, 4 h after the stressor.

After 6 weeks of treatment, fish in each system were anesthetized to determine the total biomass. Two random fish in each system were sacrificed for the determination of growth parameters, gill histology, innate immunity and oxidative stress (n=6 per group). Blood was immediately collected with a non-heparinized syringe from the caudal vein. Serum was collected after clotting overnight at 4 °C and subsequent centrifugation at 750 g for 10 min, divided into several aliquots and stored at – 80 °C. Serum osmolality was determined using a VAPRO® Vapor Pressure Osmometer (Wescor, Inc, Utah, USA). Biomass, standard length and height were measured. The whole liver was removed and weighed to calculate the liver-somatic-index. The second gill arch on the right side was removed, embedded in cassette and immediately fixed in Bouin's solution for histology analysis. The head kidney was aseptically removed, pressed through a 70 µm EASYstrainer^{TM} sterile mesh unit (Greiner Bio-One International, Kremsmünster, Austria) and suspended in ice-cold wash medium (RPMI-1640 with phenol red + 100 U mL^{-1} Penicillin-streptomycin +2 mM L-Glutamine + 25 mM Hepes buffer, 0.22 µm sterile filtered).

2.2.2. Mirror carp in a recirculating aquaculture system

The challenge of bad water quality was realized by the transient stop of water flow in all fish tanks every Monday and Thursday. Feeding was withdrawn one day prior to water stops. Simultaneous to the water stop, eight fish tanks (treatment group) received 750 µL Wofasteril® E400 (Kesla, Wolfen, Germany), resulting in a nominal PAA concentration of 1 mg L^{-1}. Meanwhile, the other 8 fish tanks (control group) received a sham treatment of 750 µL distilled water. The water flow was stopped for 3 h until it was restarted.

Carp from 2 PAA-treated tanks and 2 untreated tanks were sampled monthly after the 1^{st} water stop. Blood (n=12) was immediately collected from the caudal vein with a heparinized syringe. Hematocrit was determined by centrifuging blood in capillary tubes at 1000 g for 5 min. Plasma was isolated after centrifugation at 13 000 g for 5 min and stored at -20 °C until assayed. Osmolality of plasma was measured using OSMOMAT 030 (Gonotec, Berlin, Germany). The plasma cortisol, glucose and free fatty acid (FFA) of mirror carp were determined using the Cortisol ELISA test kit (IBL International, Hamburg, Germany), the Glucose Assay Kit (Sigma Aldrich, Munich, Germany) and the Free Fatty Acid Quantitation Kit (Sigma Aldrich, Munich, Germany), respectively, according to the manufactures' instructions.

After the blood sampling, 6 carp from each group were sacrificed. Biomass, standard length and height were measured. Head and trunk kidneys were aseptically removed, pressed through 70 µm EASYstrainer™ sterile mesh unit (Greiner Bio-One International, Kremsmünster, Austria) and suspended in ice-cold wash medium (RPMI-1640 with phenol red + 10% distilled water + 100 U mL^{-1} Penicillin-streptomycin +2 mM L-Glutamine + 25 mM Hepes buffer + 10 U mL^{-1} Heparin, 0.22 µm sterile filtered). A specific trial was performed

during the 3rd kidney sampling. Chopped kidney samples were incubated in 1 mg mL^{-1} collagenase-supplemented (Roche Diagnostics, Mannheim, Germany) wash medium at ambient temperature for 2 hours before pressed through the sterile mesh units. During the last sampling, the second gill arch on the right side was removed, embedded in cassette and immediately fixed in Bouin's solution for histology analysis.

On two random days with treatments, water samples were collected from a random tank in the control and treatment groups prior to and after the water stop, respectively. The colony forming units (CFU) in water samples were determined on agar plates using the drop plate method described by Meinelt et al. (2015).

2.3. Water parameter measurement in flow-through systems

The pH value was measured in all groups for 48 h during a pulse PAA treatment in the 1st and 4th week. The pH probe (HQ40D, Hach Lange, Düsseldorf, Germany) was fixed near the water surface before the fishes were acclimated. In the 6th week, water samples were collected from all systems at the outflow shortly before and 12 h post-feeding. Total ammonia-nitrogen (TAN), nitrite-N and nitrate-N were determined according to the methods described by von Ahnen et al. (2015). Moreover, the PAA concentration and degradation were determined in the surface water of the pulse treatment group and the continuous application group according to the DPD (N, N-diethyl-p-phenylendiamine sulfate salt) photometric method described by Pedersen et al. (2009).

2.4. Water cortisol measurement

Cortisol in the water samples was extracted immediately after sampling. The extraction was based on the procedure described by Brüning et al. (2015). Each cartridge (Sep-Pak C18 Plus, Waters, Eschborn, Gemany) was activated with 5 mL methanol and rinsed with 5 mL ultrapure water before 1 L water sample was peristaltically pumped (ISMATEC® BVP Standard, Cole-Parmer GmbH, Wertheim, Germany) through at a flow rate of 10 mL min^{-1}. Subsequently, the cartridges were rinsed with 5 mL ultrapure water, eluted with 5 mL ethyl-acetate and collected in 10-mL glass tubes. The eluted samples were evaporated in a water bath (40 °C) under a N_2 stream and subsequently stored at -20 °C until assayed. The evaporated cortisol samples were redissolved in 0.5 mL phosphate buffered saline solution (PBS + 5% ethanol + 0.1% bovine serum albumin, 0.22 µm sterile filtered) and measured with Cortisol ELISA test kits (IBL International, Hamburg, Germany) according to manufacturer's instruction.

2.5. Kidney leucocytes isolation and respiratory burst assay

Respiratory burst of kidney leucocytes was chosen as an indicator for the innate cellular immunity of fish (Ellis, 1999). The isolation of leucocytes was performed as described by Secombes (1990); Pietsch et al. (2008); Pietsch et al. (2014) with slight modifications. The cell suspensions from kidneys were centrifuged at 500 g for 10 min. The leucocyte-enriched layer was transferred with a sterile Pasteur pipette to a new sterile centrifuge tube and re-suspended in 6 mL ice-cold culture medium (same recipes as respective wash medium withdrawing heparin, the RPMI-1640 with phenol red was replaced by the RPMI-1640

without phenol red). The new suspensions were washed twice with ice-cold culture medium by repeating the centrifugation and transfer process described above. Subsequently, the cell density of each suspension was adjusted to 10^7 cells ml^{-1} with a hemocytometer (Brand, Wertheim, Germany) before the suspensions were seeded to sterile 96-well plates (Nunc Thermo Scientific, Waltham, Massachusetts, USA) with 100 µL cell suspension per well and 6 replicates. The plates were incubated in a humid chamber at 17 °C (for rainbow trout) or 22 °C (for mirror carp) overnight until the non-attached cells were removed by gently discarding the medium.

To measure the respiratory burst (NBT-assay), 100 µL fresh culture medium at ambient temperature supplemented with 1 mg mL^{-1} nitroblue tetrazolium (NBT) was added to each well. Phorbol 12-myristate 13-acetate (PMA, 1 µg mL^{-1}) was used as a stimulant. The plates were incubated in a humid chamber at previously described temperatures for 1 h (mirror carp) or 3 h (rainbow trout). Subsequently, the medium was discarded and the cells were fixed with 100% methanol and washed twice with 70% methanol. Dried wells were mixed with 100 µL dimethyl sulfoxide (DMSO) and 100 µL 2M KOH to dissolve the formazan. The optical density (OD) was read at 620 nm with a plate reader (TECAN GENios, Salzburg, Austria).

Viability of the attached leucocytes was determined simultaneously to the NBT-assay. To measure the cell viability (MTT-assay), 100 µL fresh culture medium at ambient temperature supplemented with 0.5 mg mL^{-1} thiazolyl blue tetrazolium bromide (MTT) was added to each well. The same PMA stimulant as the NBT-assay was administrated. The plates were incubated at the same condition of the NBT-assay for the same duration.

Subsequently, the medium was discarded and the cells were dried. The formazan was dissolved in 100 µL alkaline DMSO (27 µL 2M KOH dissolved in 10 mL DMSO) and the OD was read at 570 nm with a plate reader (TECAN GENios, Salzburg, Austria) after mixing.

The respiratory burst data were normalized by dividing the results of NBT-assay with the respective results of MTT-assay. The stimulation factor of PMA was calculated by dividing the normalized respiratory burst data of PMA-stimulated leucocytes with those of unstimulated leucocytes.

2.6. Histology analysis of gill

After the gill histology samples were fixed in Bouin's solution for 24 h, the Bouin's solution was discarded and replaced with 70% ethanol. The 70% ethanol was daily refreshed for 3 times. Gill samples were then dehydrated in a Shandon™ Excelsior™ Tissue Processor before they were manually embedded in paraffin blocks. Gill samples in paraffin blocks were decalcified for five to eight hours until sectioned with a rotary microtome in thickness of 3.5 µm. Serial sections were transferred to a water bath (45°C), placed on slides and dried on a heating plate (45°C). Finally, all slides were stained with hematoxylin and eosin. Cover slips were glued on the slides with Roti®-Histokitt II (Carl Roth).

All stained sections were evaluated under a light-microscope at x60 magnification. For each fish, 10 secondary filaments from the inner section on 6 slides were analyzed. Hyperplasia of the primary filament and secondary filament was evaluated in 3 degrees of severity, which was defined by the number of layers of epithelial cells: 2 to 3 was considered as 'minimal', 4 to 7 was considered as 'moderate' and ≥ 8 was considered as 'severe'. Similar

severities of eosinophilic granulocytes aggregation were defined based on the cell number: ≤ 4 was considered as minimal, 5-8 was considered as moderate and ≥ 9 was considered as severe. For each severity, the number of cases (affected filament) was noted, and multiplied by the severity factor to quantify the alteration. The severity factor of 'minimal', 'moderate' and 'severe' was defined as 1, 2 and 3, respectively. Finally, the total alteration was determined by the summation of quantified alterations of all severities.

2.7. Measurement of total antioxidant capacity and total free radical

Total antioxidant capacity (TAC) was quantified in the gill, liver and serum of rainbow trout using a commercially available kit (Sigma, Steinheim, Germany). Homogenates of the gill and liver were prepared following the protocol provided in the assay kit. Trolox, a water-soluble vitamin E analog, served as the standard and antioxidant capacity was expressed in Trolox equivalents. Total antioxidant capacity was quantified in the gill, liver and serum using a commercially available kit (Sigma, Steinheim, Germany). Homogenates of the gill and liver were prepared following the protocol provided in the assay kit. Trolox, a water-soluble vitamin E analog, served as the standard and antioxidant capacity was expressed in Trolox equivalents. The level of total free radicals, reactive oxygen species (ROS)/reactive nitrogen species (RNS), in the abovementioned organs and biological fluid was determined using a commercial kit (Cell Biolabs, California, USA). The ROS/RNS was quantified fluorometrically and expressed in relation to 2', 7'-dichlorodihydrofluorescein (DCF) standard.

2.8. Measurement of innate humoral immunity in rainbow trout

Total serum protein concentration was quantified using bovine serum albumin as a standard (Thermo scientific, Illinois, USA). The inhibition of trypsin activity was employed to quantify the antiprotease activity (Hanif et al., 2004). Percentage of inhibition was calculated by comparing it to 100 % control. Myeloperoxidase was measured following previously described protocol (Quade and Roth, 1997), with modifications (Lazado et al., 2015), using 3,30,5,50-tetramethyl benzidine hydrochloride as a reaction substrate. Unit of activity was expressed as OD at 450 nm. Lysozyme activity was quantified by a turbidimetric method (Parry Jr et al., 1965), following a modified protocol for 96-well microplate reaction (Sitjà-Bobadilla et al., 2008). A unit of lysozyme activity was defined as the amount of enzyme that caused a decrease in absorbance of 0.001 per minute at 450 nm. A kinetic reaction assay using *p*-nitrophenyl phosphate as a substrate was employed to measure the level of alkaline phosphatase (Ross et al., 2000). One unit of activity was defined as the amount of enzyme required to release 1 mmol of *p*-nitrophenol product in 1 min. Esterase activity was determined using *p*-nitrophenyl myristate as substrate (Ross et al., 2000). Enzyme activity was expressed similarly as with alkaline phosphatase (Lazado et al., 2016). All OD measurements were conducted in a microplate reader (TECAN GENios, Salzburg, Austria).

2.9. Statistics

The cortisol concentrations, growth parameters, cellular/humoral innate immunity, total quantified gill alteration, oxidative stress markers and water parameters were

compared via independent T-test (2 treatment groups) or one-way ANOVA with Tukey post-hoc test (3 treatment groups). The presence of gill alterations at different severities was compared via two-way ANOVA.

Data were square-rooted or logarithm transformed, if normality failed. In case of heterogeneous variance, a Welch's ANOVA with Dunnett's T-3 post-hoc test was performed instead of a standard ANOVA. All analysis was performed on SPSS Statistics 21 (IBM, Chicago, USA).

The water cortisol after the additional stressor (dipnet harassment) was analyzed with GraphPad Prism® 7 (GraphPad Software, California, USA). The increase of cortisol along time was interpreted as a linear regression. The slope and intercept of different groups were compared.

3. Results

3.1. Rainbow trout in flow-through systems with optimal water quality

3.1.1. Water cortisol

The water cortisol concentration in the control treatment group indicated diurnal fluctuation during the sampling period. The concentration slightly increased from 5.03 ± 0.61 ng L^{-1} in the morning, peaked at noon (5.61 ± 0.14 ng L^{-1}) and decreased in the afternoon (Figure 3). This fluctuation was repeated throughout the 4 weeks of measurement. The water cortisol concentration from the continuous treatment group showed a similar pattern as the control group. In contrast, the water cortisol of the pulse treatment group showed a significant increase after the 1st PAA application. The water cortisol concentration remained higher than the other two groups for hours, reaching levels up to 27.88 ± 5.98 ng L^{-1}. Moreover, fish showed behavioral reactions to the pulse PAA treatments. They became more active in swimming rather than 'aligned' in the radial flow. In the following weeks, the increase of water cortisol in the pulse treatment group became progressively milder and negligible (Figure 3). The intensity and the duration of the behavioral reaction of fish were also decreasing over time.

Noteworthy, there were a few fish that showed either aggressive or evading behavior regardless of treatment group and time. Some were chasing and biting each other, while the rest were close to the bottom of the tank with their head tilting slightly downward. In contrast, fish in other replicate tanks remained peaceful in schools. These fish introduced a prolonged higher water cortisol concentration and a different pattern than the other replicate systems within the same group (Figure 3).

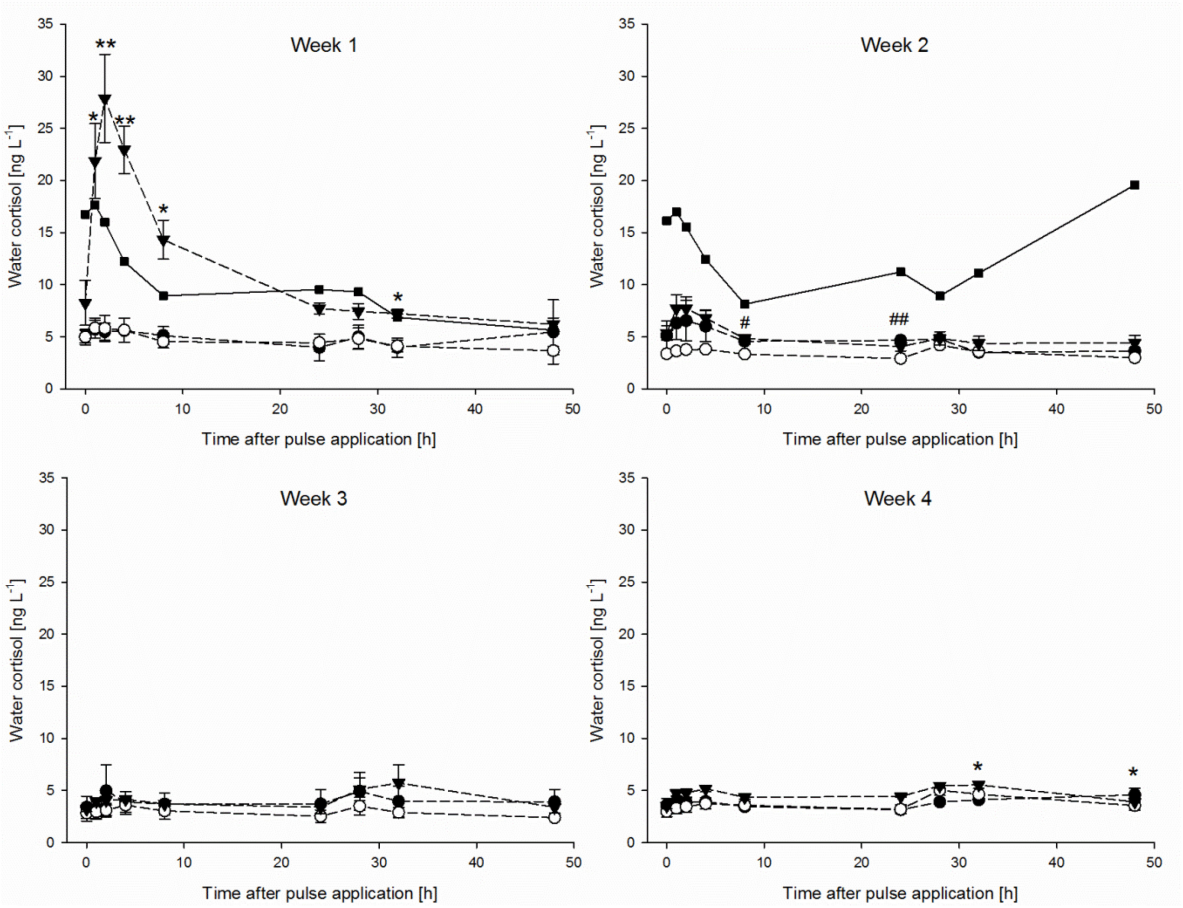

Figure 3 The mean water cortisol concentration (n=3) from the control group (●), the continuous treatment group (○) and the pulse treatment group (▼) based on weekly measurements during the first 4 weeks. ■: fish that showed aggressive or evading behavior in the control group.

Error bars indicate the standard error.

* and ** indicate significant difference between the control and the pulse treatment group, P< 0.05 and 0.01, respectively.

and ## indicate significant difference between the control and the continuous treatment group, P< 0.05 and 0.01, respectively.

The water cortisol concentration of all groups increased after the application of the additional stressor (dipnet harassment for 90 s) in the 5th week. The proportional increase was similar for all treatment groups (*df*=8, P=0.578). Moreover, the slope and intercept of the cortisol-time linear regression were similar in all treatment groups (*df*=8, P=0.759 and 0.0617, respectively; Figure 4).

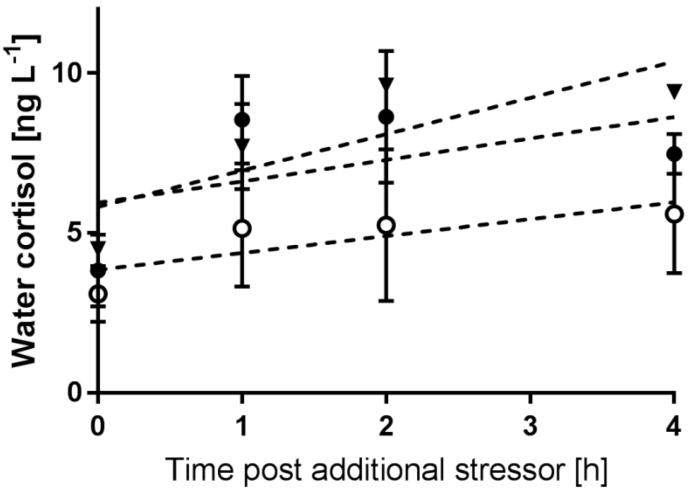

Figure 4 The mean water cortisol concentration from the control group (●), the continuous treatment group (○) and the pulse treatment group (▼) immediately after an additional stressor (dipnet harassment) in the 5th week.

Error bars indicate the standard error.

Dotted lines indicate the interpreted linear regression between time and water cortisol.

3.1.2. Growth

No mortality occurred during the experimental period. No uneaten pellets were observed by the daily inspections throughout the experiment. The total growth rate and feed conversion ratio were the same for all groups (*df*=17, P=0.813 and 0.907, respectively;

Table 1). The length/height ratio and liver-somatic-index of sampled fish were the same for all groups (*df*=17, P=0.771 and 0.824, respectively; Table 1).

Table 1 Growth parameters (mean ± standard deviation) of rainbow trout in all treatment groups after 6 weeks.

Parameters	**Control**	**Continuous treatment**	**Pulse treatments**	**P-value (2-tail)**
Initial biomass [kg]	2.14 ± 0.03	2.13 ± 0.02	2.12 ± 0.03	0.694
Final biomass [kg]	3.52 ± 0.02	3.51 ± 0.012	3.51 ± 0.031	0.467
Growth rate [%]	64.5 ± 1.88	64.9 ± 2.1	65.4 ± 0.9	0.813
FCR	0.70 ± 0.012	0.70 ± 0.02	0.70 ± 0.001	0.907
Liver somatic index	0.012 ± 0.001	0.012 ± 0.001	0.011 ± 0.001	0.865
Length/height ratio	3.86 ± 0.11	3.85 ± 0.20	3.79 ± 0.22	0.853

FCR: Feed conversion ratio

3.1.3. Respiratory burst of head kidney leucocytes

Head kidney leucocytes of rainbow trout from all treatment groups showed similar respiratory burst (*df*=17, P=0.663 and 0.364 for unstimulated and PMA-stimulated leucocytes, respectively; Figure 5). The stimulation factor (SF) of PMA on respiratory burst of head kidney leucocytes was also similar in all groups (*df*=17, P=0.714).

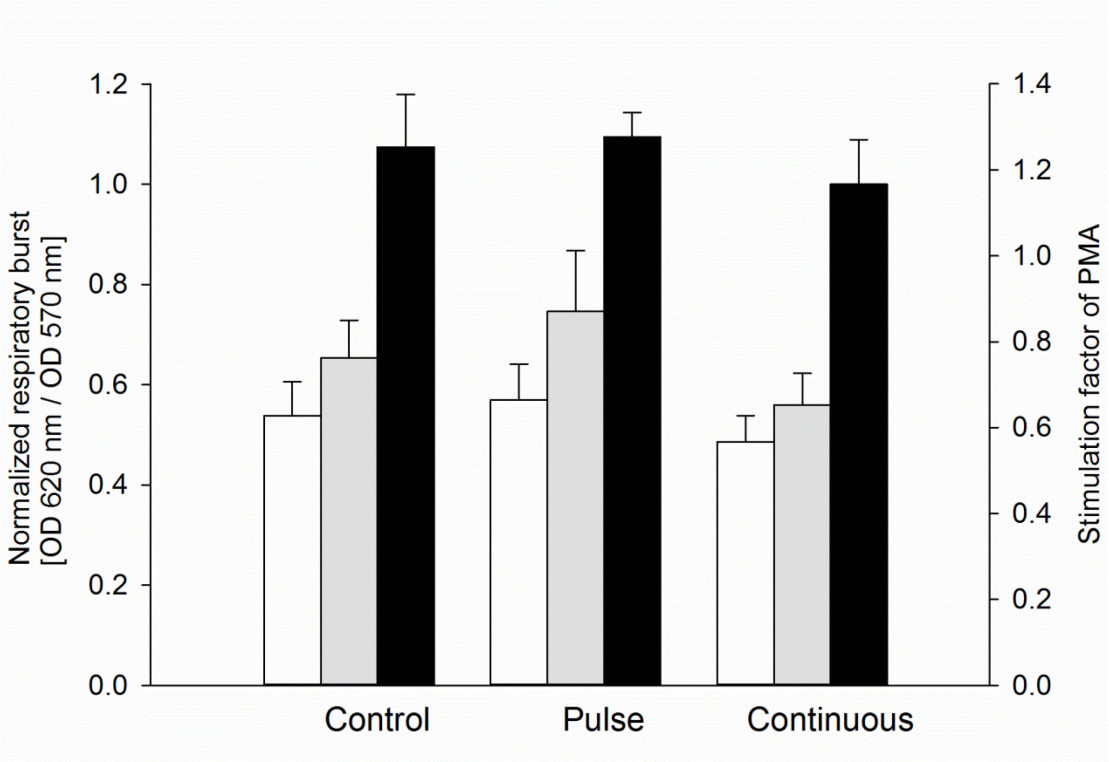

Figure 5 The respiratory burst of head kidney leucocytes of rainbow trout receiving no PAA treatment (control), pulse PAA treatments (Pulse) and continuous PAA treatment (Continuous).

Open bars indicate unstimulated leucocytes.

Grey bars indicate leucocytes stimulated with 1 µg mL^{-1} PMA.

Black bars indicate the stimulation factor of PMA on the leucocyte respiratory burst.

Error bars indicate the standard error.

3.1.4. Innate humoral immunity

The activity of esterase, alkaline phosphatase, lysozyme and myeloperoxidase in serum were similar in rainbow trout receiving pulse PAA treatments, continuous PAA treatment and no PAA treatment (*df*=17, P=0.192, 0.316, 0.819 and 0.124, respectively; Table 2). The total serum protein and the serum osmolality had no difference among groups, too (*df*=17,

P=0.542 and 0.075, respectively; Table 2).

Table 2 Parameters (mean ± standard deviation) in serum related to innate humoral immunity and osmolality of rainbow trout from all treatment groups.

	Control	**Pulse treatment**	**Continuous treatment**	**P-value (2-tail)**
Lysozyme [U mL^{-1}]	1012.73 ±77.80	960.33 ± 92.29	958.93 ± 234.63	0.819
AP [U mL^{-1}]	18.88 ± 5.99	18.00 ± 4.42	14.70 ± 3.88	0.316
Esterase [U mL^{-1}]	0.23 ± 0.04	0.17±0.07	0.23 ± 0.05	0.192
TSP [mg mL^{-1}]	35.57 ± 2.41	38.51 ± 4.97	37.07 ± 3.51	0.542
MP [OD 450 nm]	0.35±0.13	0.55±0.30	0.27 ± 0.29	0.124
SO [mmol Kg^{-1}]	290 ± 5	306 ± 14	307 ± 13	0.075

AP: alkaline phosphatase

TSP: total serum protein

MP: myeloperoxidase

SO: serum osmolality

The antiprotease activity in serum of rainbow trout receiving continuous PAA treatment was lower than that of control rainbow trout (*df*=17, P=0.005; Figure 6). In contrast, the serum antiprotease activity of rainbow trout receiving pulse PAA treatments was similar to that of control rainbow trout (*df*=17, P=0.091).

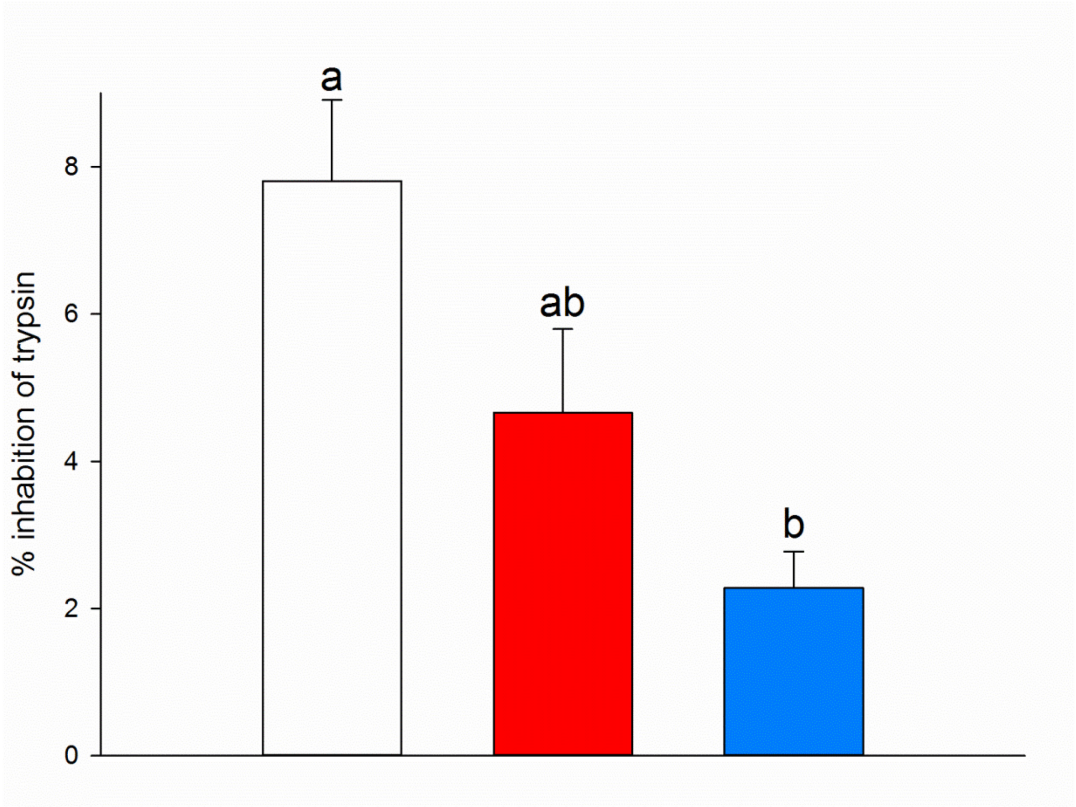

Figure 6 The activity of antiprotease in serum of rainbow trout receiving no PAA treatment (blank bar), pulse PAA treatments (red bar) and continuous PAA treatment (blue bar). Error bars indicate the standard error.

The letters 'a', 'b', and 'ab' indicate homogenous subsets base on one-way ANOVA analysis.

3.1.5. Total antioxidant capacity and total free radical

The total antioxidant capacity (TAC) in liver showed no significant difference among the pulse PAA-treated, continuous PAA-treated and untreated fish (*df*=17, P=0.165; Figure 7). In serum, however, the TAC was highest in the pulse PAA-treated fish and lowest in the untreated fish (*df*=17, P<0.001). In gill, the TAC was similar between the untreated and pulse PAA-treated fish (*df*=17, P=0.999), while a higher TAC was observed in the continuous PAA-treated fish (*df*=17, P=0.036). The baseline TAC measured in the control group was

highest in gill, intermediate in liver and lowest in serum.

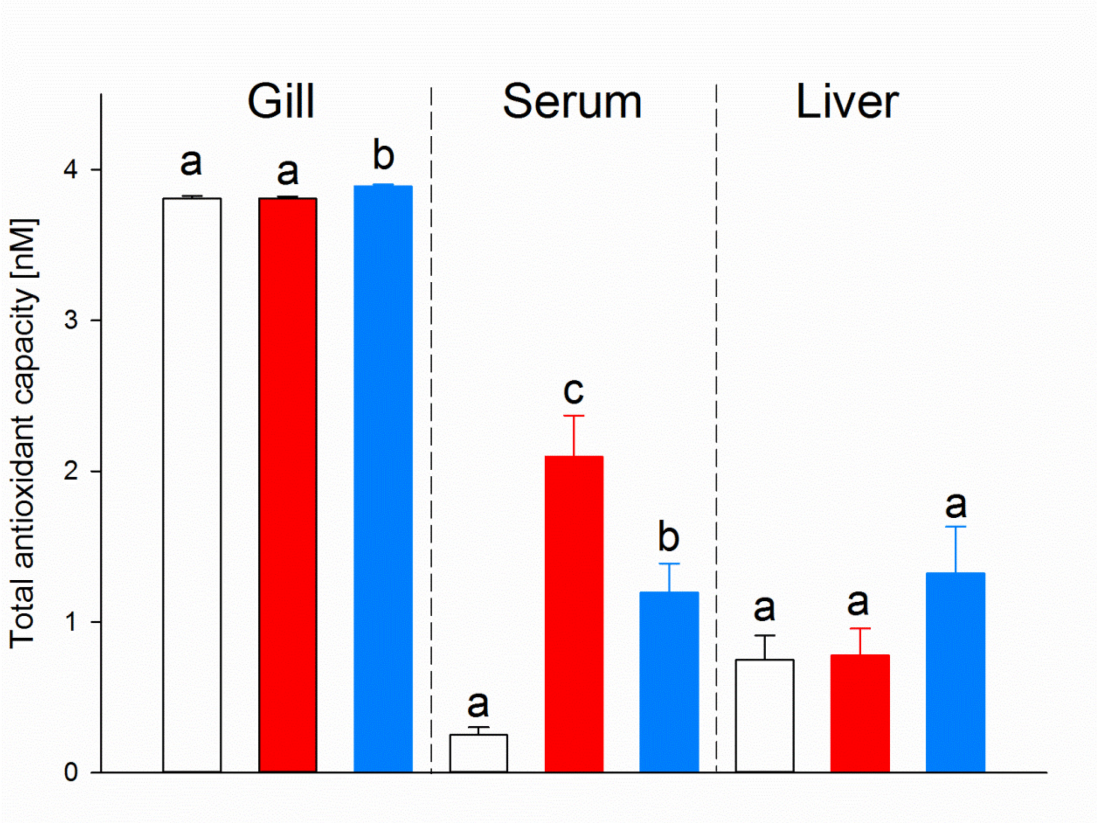

Figure 7 The total antioxidant capacity in gill, serum and liver of rainbow trout receiving no PAA treatment (blank bars), pulse PAA treatments (red bars) and continuous PAA treatment (blue bars).

Error bars indicate the standard error.

The letters 'a', 'b', and 'c' indicate homogenous subsets in respective organs base on one-way ANOVA or Welch's ANOVA analysis.

The total free radical concentration (ROS/RNS) in liver was similar in the pulse PAA-treated, continuous PAA-treated and untreated fish (*df*=11, P=0.737; Figure 8). In serum, the ROS/RNS concentration of the pulse PAA-treated fish was higher than the untreated fish (*df*=11, P=0.03), while the ROS/RNS concentration of the continuous PAA-treated fish was

similar to the untreated and pulse PAA-treated fish (*df*=11, P=0.089 and 0.788, respectively). In gill, the ROS/RNS concentration of the continuous PAA-treated fish was higher than the untreated fish (*df*=11, P=0.02), while the ROS/RNS concentration of the pulse-treated fish was similar to the untreated and continuous-treated fish (*df*=11, P=0.936 and 0.326, respectively). The baseline ROS/RNS concentration measured in the control group was lowest in gill, intermediate in serum and highest in liver.

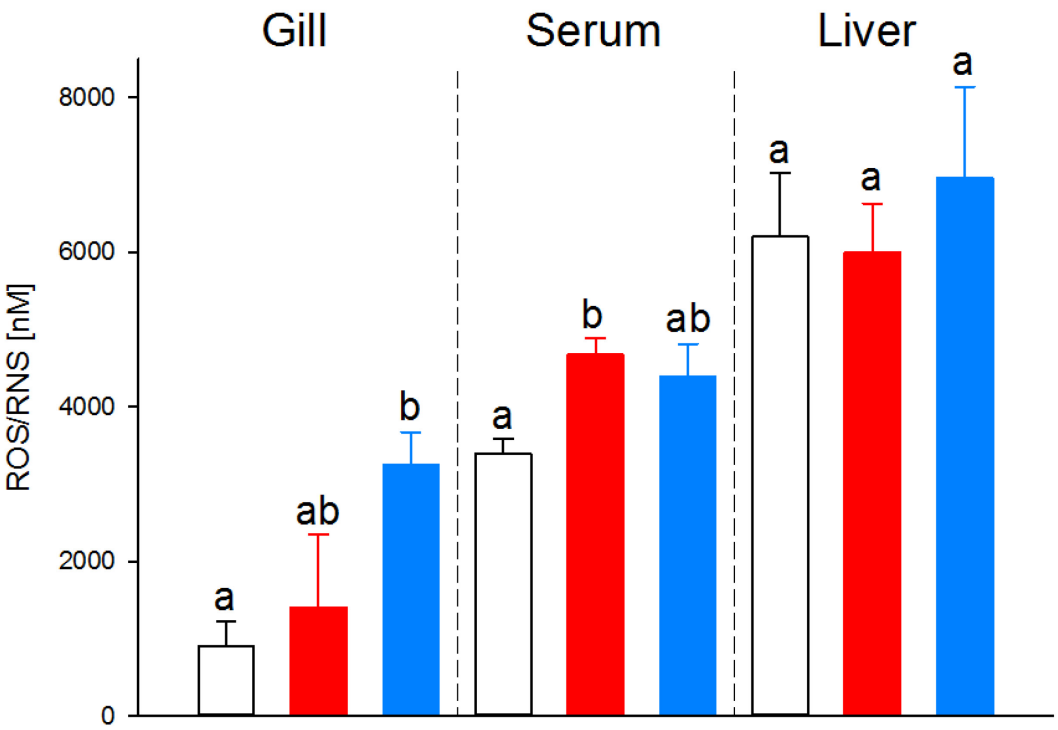

Figure 8 The level of total free radical (ROS/RNS) in gill, serum and liver of rainbow trout receiving no PAA treatment (blank bars), pulse PAA treatments (red bars) and continuous PAA treatment (blue bars).

Error bars indicate the standard error.

The letters 'a', 'b', and 'ab' indicate homogenous subsets in respective organs base on one-way ANOVA or Welch's ANOVA analysis.

3.1.6. Gill alteration

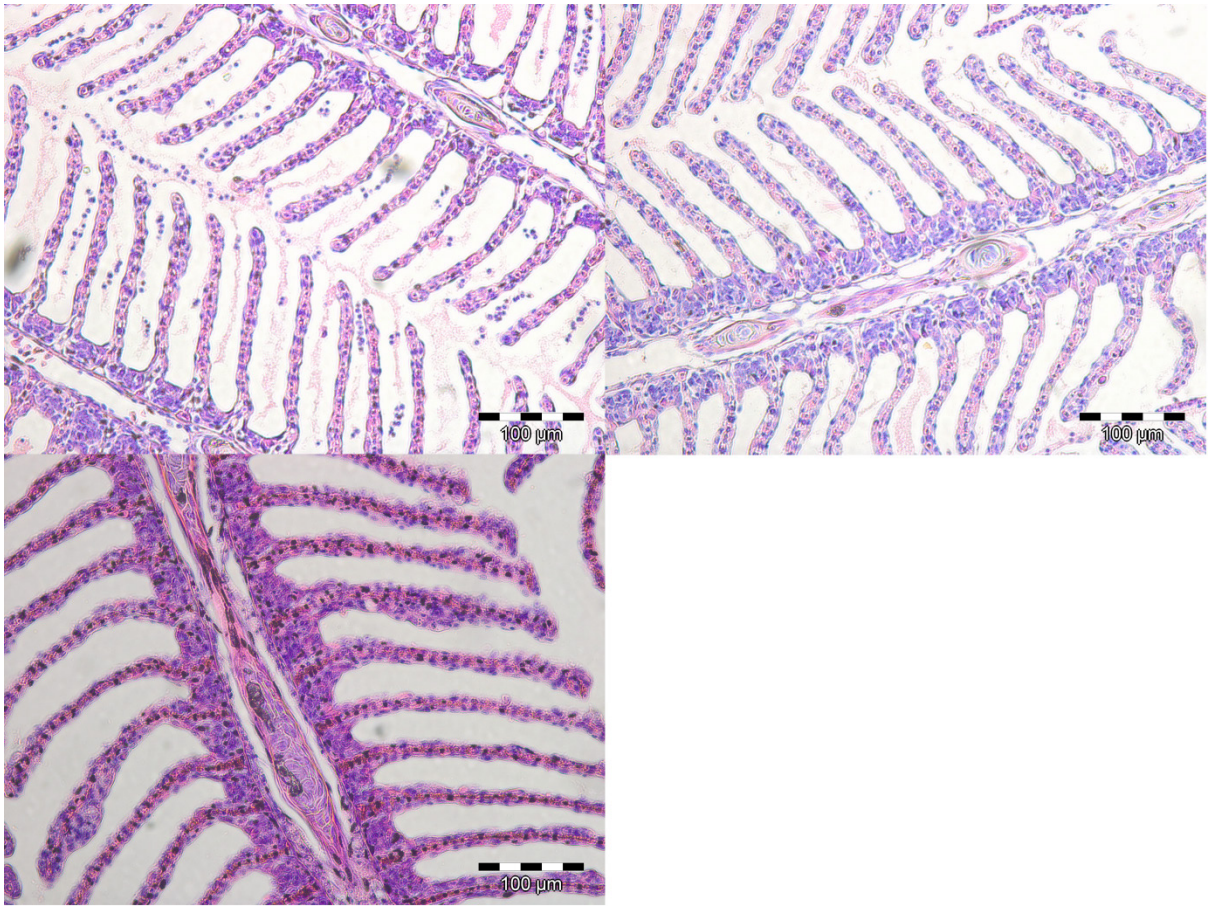

Figure 9 Selected gill histology slides of rainbow trout from the control group (upper left), pulse treatment group (upper right) and continuous treatment group (lower left) under x60 magnification.

Gill histological samples of rainbow trout from all groups were in a very good condition, and the only observed gill alteration was hyperplasia (Figure 9). No 'severe' hyperplasia was observed in all groups. The 'moderate' hyperplasia was hardly present in all treatment groups. In contrast, the 'minimal' hyperplasia was dominantly present, and more frequently observed in the pulse treatment group than the control group (df=17, P=0.025; Figure 10). Regardless of the severity, the pulse treatment group had significantly more hyperplasia

than the control group, too (*df*=17, P=0.048). In contrast, the continuous treatment group had similar frequency of hyperplasia to the control and pulse treatment group (*df*=17, P=0.857 and 0.142, respectively). In terms of total quantified gill alteration, no difference was observed among all groups (*df*=17, P=0.18; Figure 11).

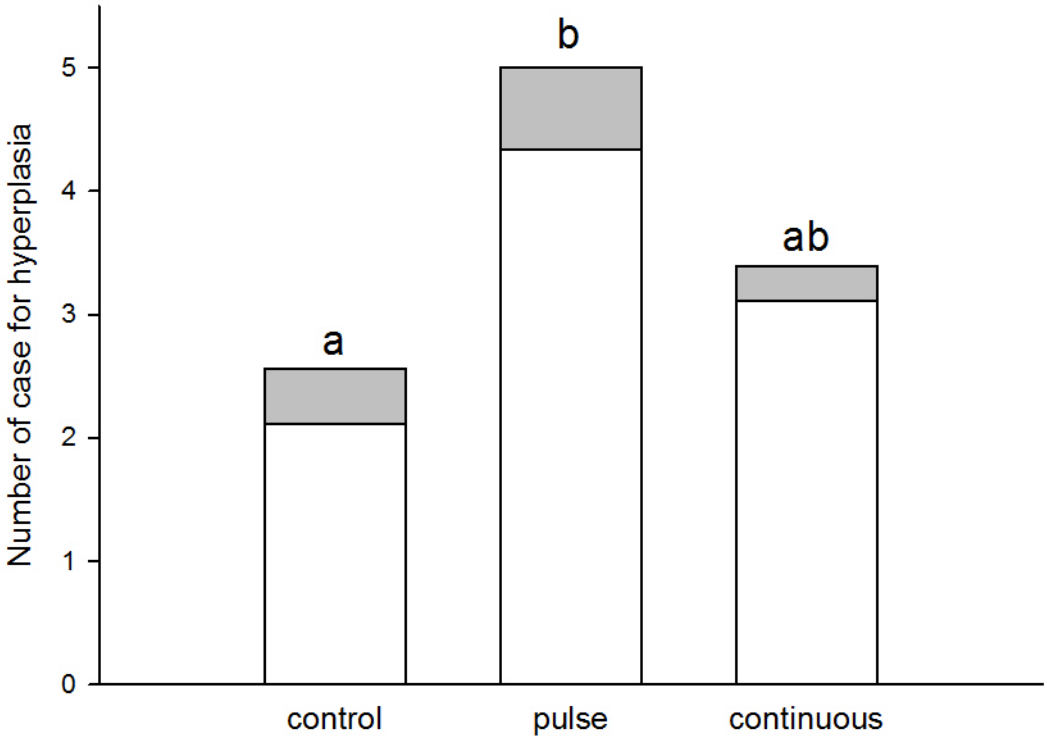

Figure 10 Average presence (number of cases) of 'minimal' (open bars) and 'moderate' (grey bars) hyperplasia in gill samples from the control, pulse treatment and continuous treatment groups. 'Severe' hyperplasia was absent in all groups.

The letters 'a', 'b' and 'ab' indicate homogenous subsets based on two-way ANOVA analysis.

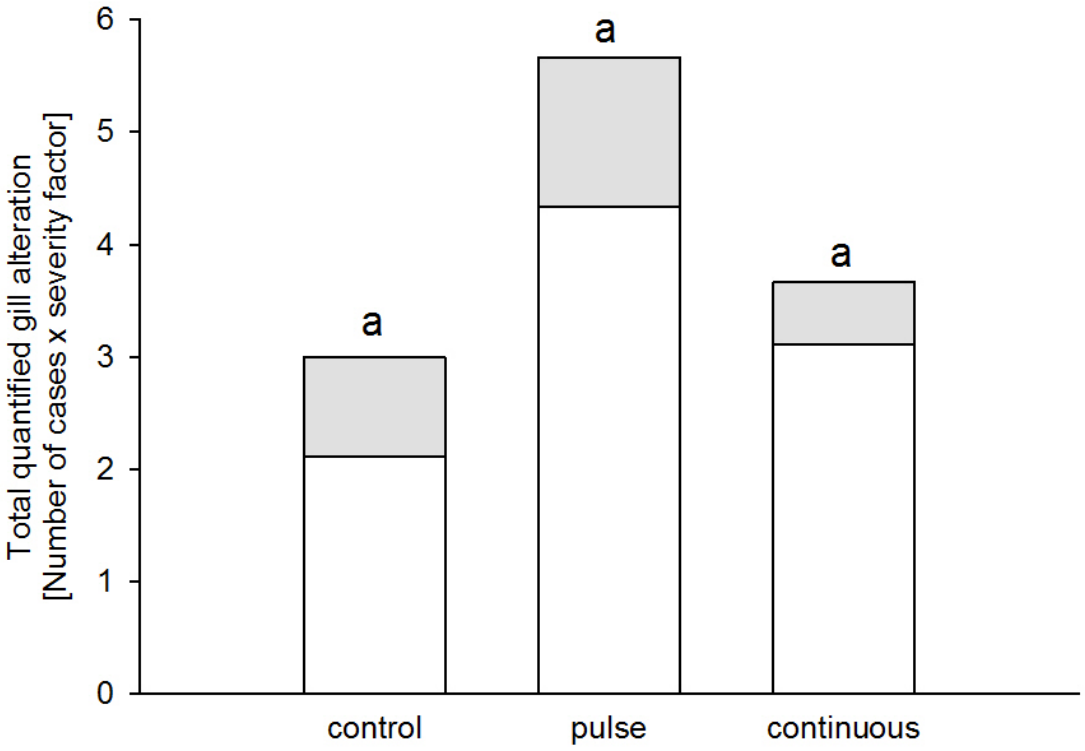

Figure 11 The average total quantified gill alteration of rainbow trout from the control, pulse treatment and continuous treatment groups.

Open bars indicate the 'minimal' severity, and grey bars indicate the 'moderate' severity.

The letter 'a' indicates homogenous subsets based on Welch's ANOVA analysis.

3.1.7. Water quality

The pH showed daily fluctuation of ≤ 0.2 in all groups. The common daily change of pH was that it slightly decreased during the feeding, and increased afterwards. The pulse PAA treatment induced a transient pH decrease immediately after the application, which was similar to the pH decrease during feeding (Figure 12). The pH of the control group decreased about 0.2 from the 1st week to the 4th week, while that of the continuous application group slightly increased. In contrast, the pH of the repeated pulse group remained the same.

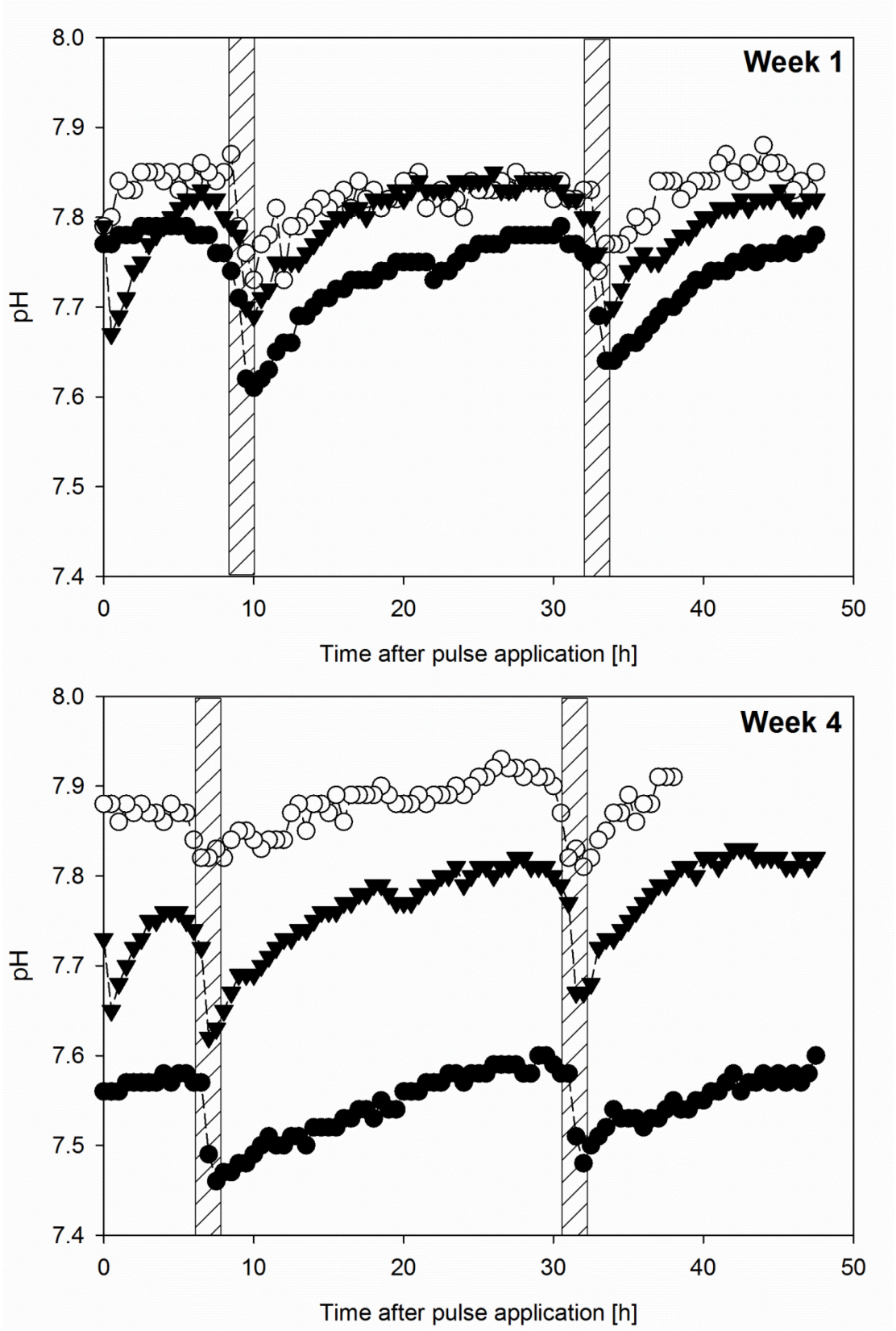

Figure 12 The pH values of the control group (●), the continuous treatment group (○) and the pulse treatment group (▼) post pulse PAA application in the 1st and 4th week. The bars

with stripes indicate the time of feeding starting at 17:30.

Compared to the control group, it was observed that the biofilm on the inner surface of the fish tank was nearly completely removed by the pulse PAA treatments. Opposite to this, enhanced biofilm formation was observed in all three systems receiving continuous application PAA addition (Figure 13).

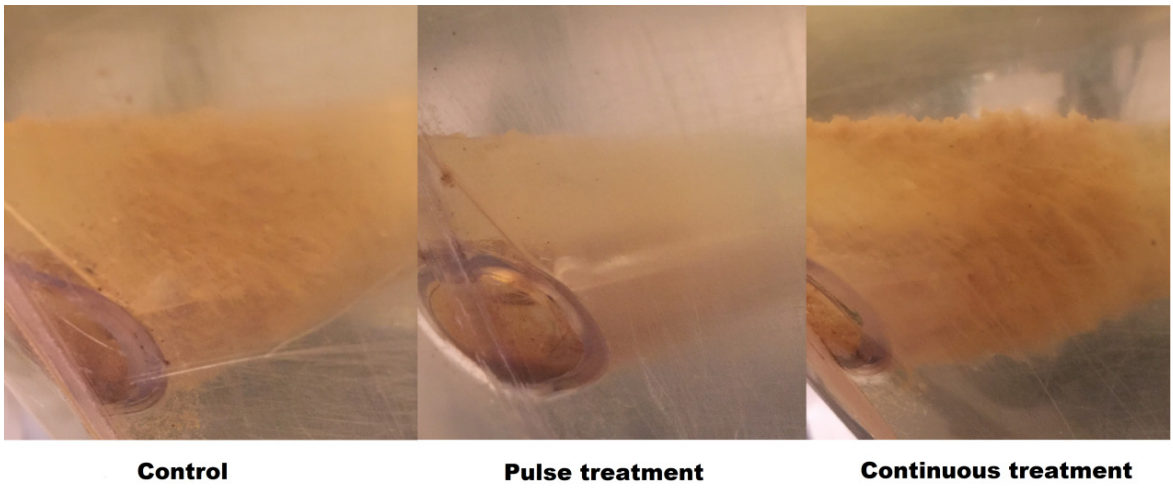

Figure 13 Biofilm on the inner side of a fish tank in the control group, the pulse treatment group and the continuous application group after 6 weeks of treatments.

Temperature in each tank was equal and remained constant at 13±0.2 °C. The dissolved oxygen concentration was highest in the pulse treatment group and lowest in the control group (*df*=377, P<0.001; Figure 14).

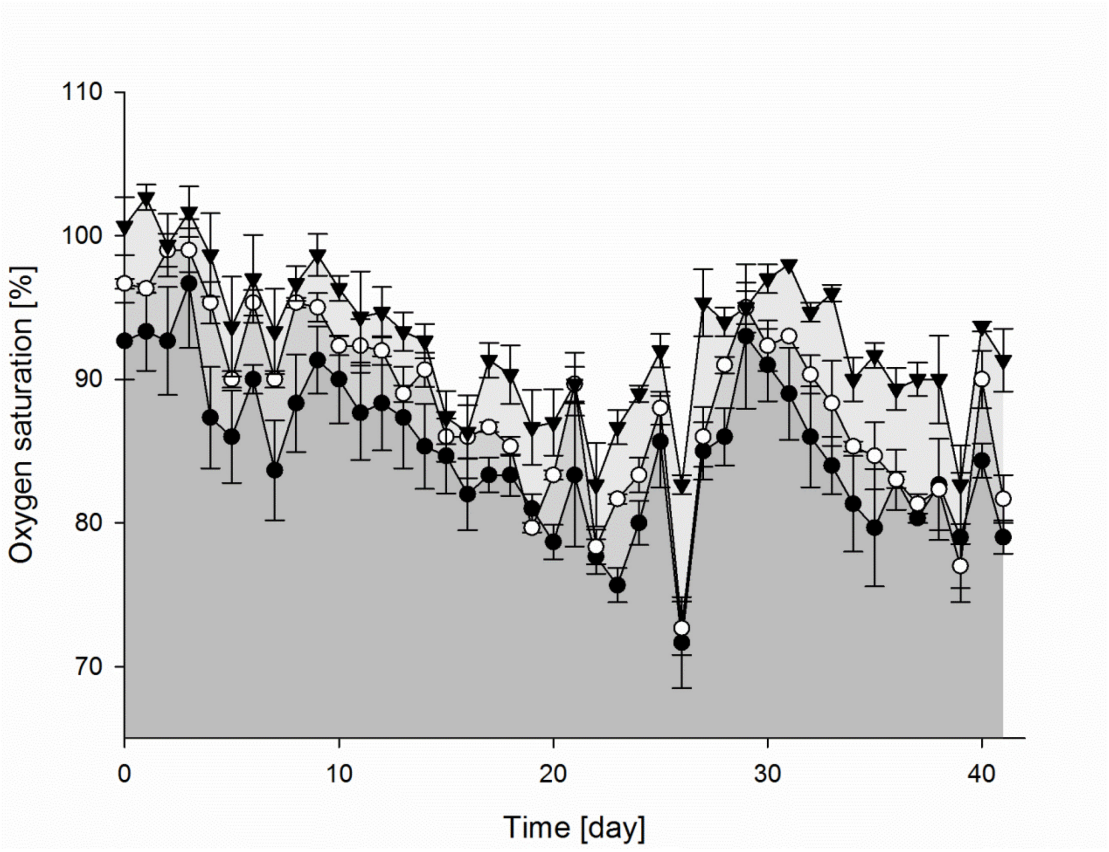

Figure 14 The average dissolved oxygen in fish tanks of the control group (●), the continuous treatment group (○) and the pulse treatment group (▼) during the experiment. Error bars indicate the standard error.

Regardless of the sampling time, the continuous treatment group and the control group had similar concentrations of dissolved TAN, nitrite-N and nitrate-N (*df*=8, before feeding: P=0.67, 0.84 and 0.92, respectively; 12 h after feeding: P=0.75, 0.90 and 0.89, respectively; Figure 15). In contrast, the pulse treatment group had significantly elevated TAN/nitrite-N and lower nitrate-N than the control group (*df*=8, before feeding: P=0.024, P<0.001 and P=0.01, respectively; 12 h after feeding: P=0.04, 0.002 and 0.005, respectively).

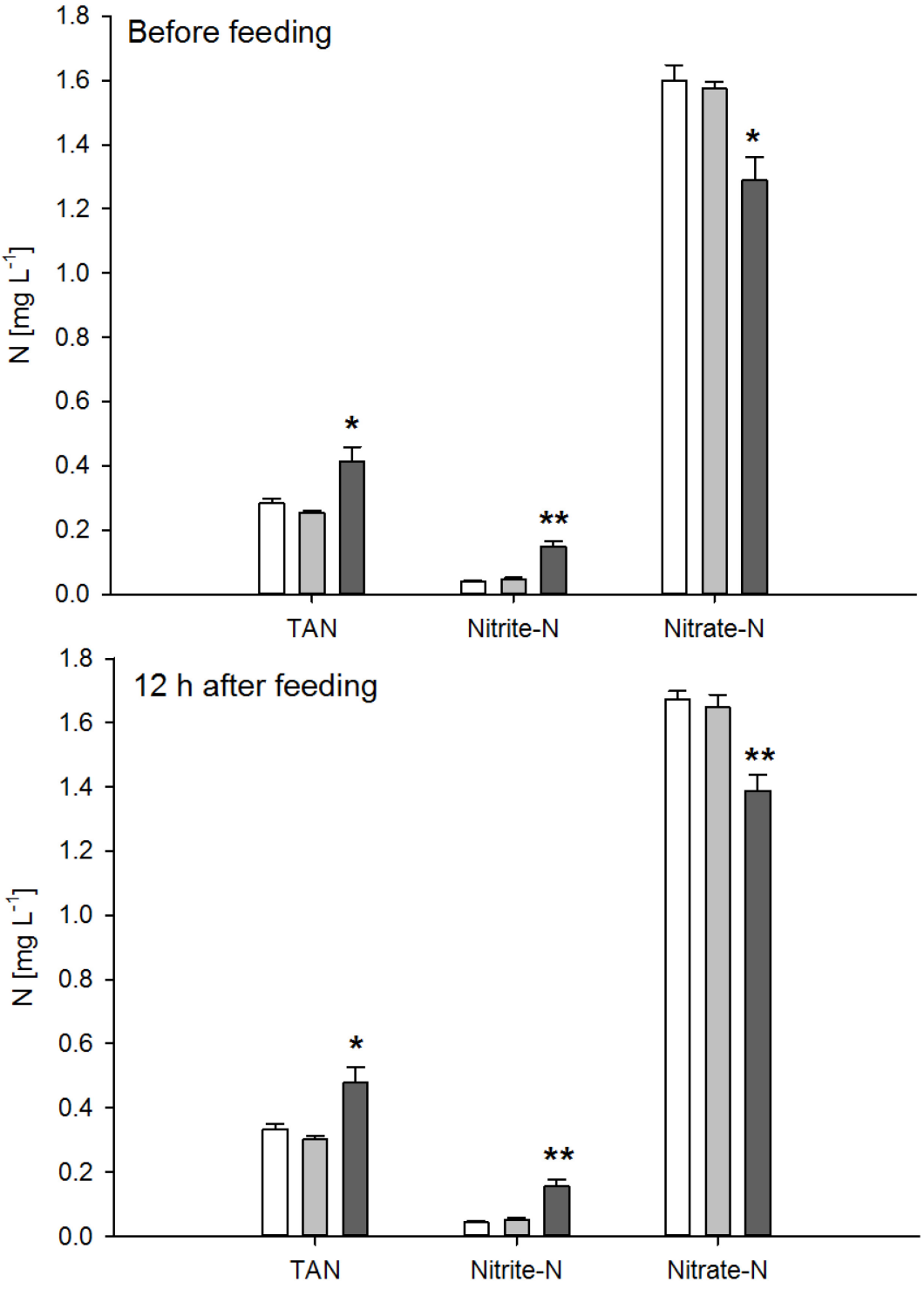

Figure 15 The dissolved total ammonium-nitrogen (TAN), nitrite-N and nitrate-N in tanks of the control group (open bars), continuous application group (grey bars) and repeated pulse group (black bars) before and 12 h post feeding on a non-pulse-treatment day in the 6th week.

Error bars indicate the standard error.

* and ** indicate significant difference from the control group (P<0.05 and 0.01, respectively).

The pulse treatment resulted in about 0.7 mg L^{-1} PAA when sampled 5 min after application. PAA concentration was exponentially reduced and the complete degradation was achieved within 5 hours after application. In systems receiving continuous PAA treatment, the nominal and subsequent PAA concentration remained below 0.01 mg L^{-1}, which was below the detection range of the described method applied (Figure 16).

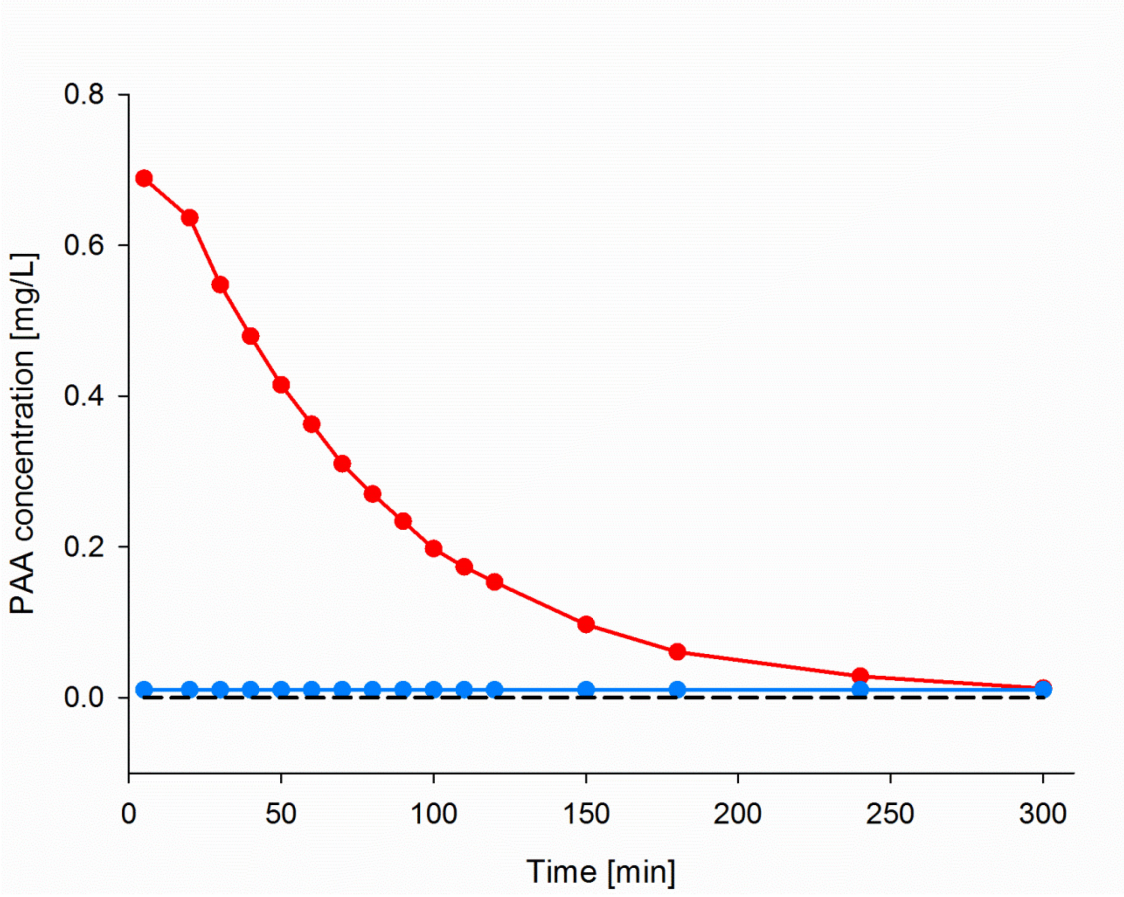

Figure 16 The concentration changes of PAA after pulse (red dots/line) and continuous (blue dots/line) applications. Black dotted line indicates the PAA concentration of 0 mg L^{-1}.

3.2. Carp in a RAS

3.2.1. Growth

No mortality was observed in both groups throughout the experiment. The average biomass and length/height ratios of the carp in the control and PAA-treated groups were similar within the experimental period (Table 3). Gonadal development was observed in sacrificed fish during the experiment.

Table 3 The average biomass and length/height ratios (mean ± standard deviation; n=6) of the mirror carp receiving transient water stops alone (control) or simultaneous 1 mg L^{-1} PAA treatments (PAA-treated) during periodic samplings.

	Control	PAA-treated	P value (2-tailed)
Biomass [g]			
0 month	1256 ± 405.5	1250.3 ± 278	0.978
1 month	1266.1 ±315.5	1339.4 ± 203.4	0.643
2 months	1158.9 ± 135	1295.1 ± 257	0.277
3 months	1244.8 ± 178.7	1445.4 ± 228.4	0.121
Length/height ratio			
0 month	3.337 ± 0.267	3.619 ± 0.322	0.129
1 month	3.463 ± 0.205	3.386 ± 0.131	0.458
2 months	2.429 ± 0.0549	2.511 ± 0.0761	0.058
3 months	2.410 ± 0.0901	2.465 ± 0.0827	0.294

3.2.2. Hematic parameters

The hematocrits of the mirror carp in the control and treatment groups were mostly similar. The only exception was during the 3rd sampling, mirror carp receiving transient water stops alone had a lower hematocrit than those receiving simultaneous 1 mg L^{-1} PAA treatments (*df*=10, P=0.037; Figure 17). The plasma osmolality of the carp was constantly similar in the control and treatment groups (Table 4).

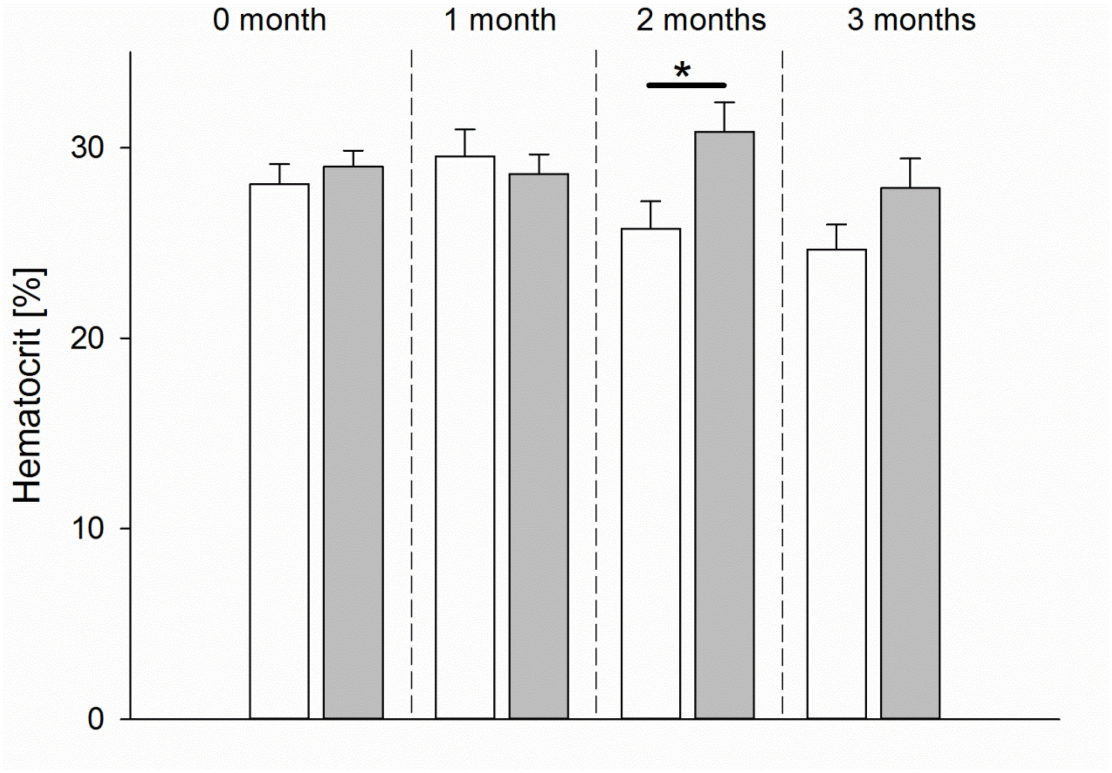

Figure 17 The hematocrits of the mirror carp receiving transient water stop alone (control group, open bars) and simultaneous 1 mg L^{-1} PAA treatment (treatment group, grey bars) during periodic samplings.

*: P<0.05 according to the T-test between the control and PAA-treated groups.

Error bars indicate the standard error.

Table 4 The average plasma osmolality (mean ± standard deviation; n=12) of the mirror carp receiving transient water stops alone (control) or simultaneous 1 mg L^{-1} PAA treatments (PAA-treated) during periodic samplings.

	Control	PAA-treated	P value (2-tailed)
Plasma osmolality [mOsmol Kg^{-1}]			
0 month	279±13.5	286±20.4	0.391
1 month	282±6.2	287±6.0	0.0626
2 months	287±7.1	283±6.8	0.183
3 months	287±10.1	282±8.9	0.185

3.2.3. Plasma cortisol, glucose and free fatty acid

During the first 2 samplings, the mirror carp receiving transient water stops alone had higher plasma cortisol than those receiving simultaneous 1 mg L^{-1} PAA treatments (*df*=22, P=0.024 and 0.033, respectively; Figure 18). During the 3rd sampling, the PAA-treated carp had similar plasma cortisol to those without PAA treatments (*df*=22, P=0.297). During the 4th sampling, the PAA-treated carp had higher plasma cortisol than those without PAA treatments (*df*=22, P=0.004). The plasma cortisol concentration of the mirror carp from both groups showed a decreasing trend along samplings.

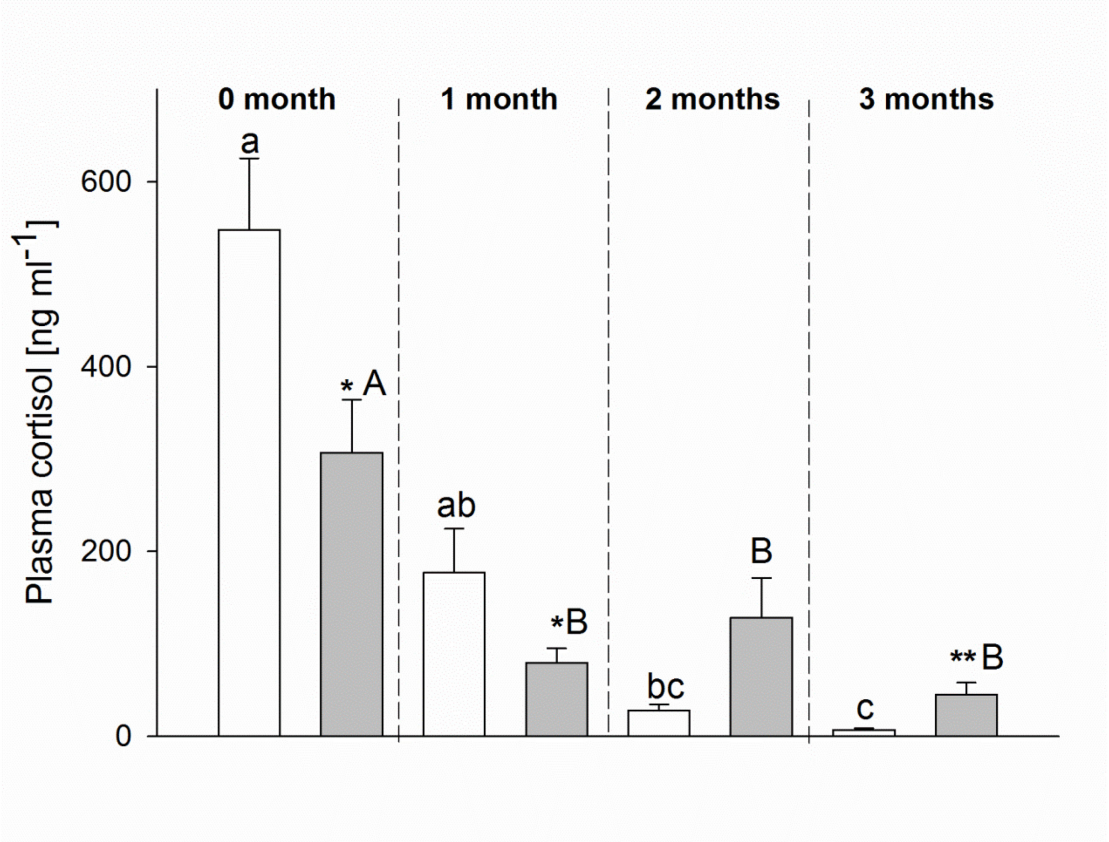

Figure 18 The average plasma cortisol concentration of the mirror carp receiving transient water stops alone (control group, open bars) or simultaneous 1 mg L^{-1} PAA treatments (treatment group, grey bars) along periodic samplings.

* and **: P<0.05 and 0.01, respectively, according to T-test between control and PAA-treated groups during each sampling

The letters a, ab, bc and c indicate homogenous subsets of the control group along time.

The letters A and B indicate homogenous subsets of the treatment group along time.

Error bars indicate the standard error.

The plasma glucose and free fatty acid concentrations were always similar in mirror carp from both groups (*df=22*, P>0.05; Figure 19 and 20). Moreover, the plasma glucose and free fatty acid of mirror carp from both groups showed a decreasing trend along samplings.

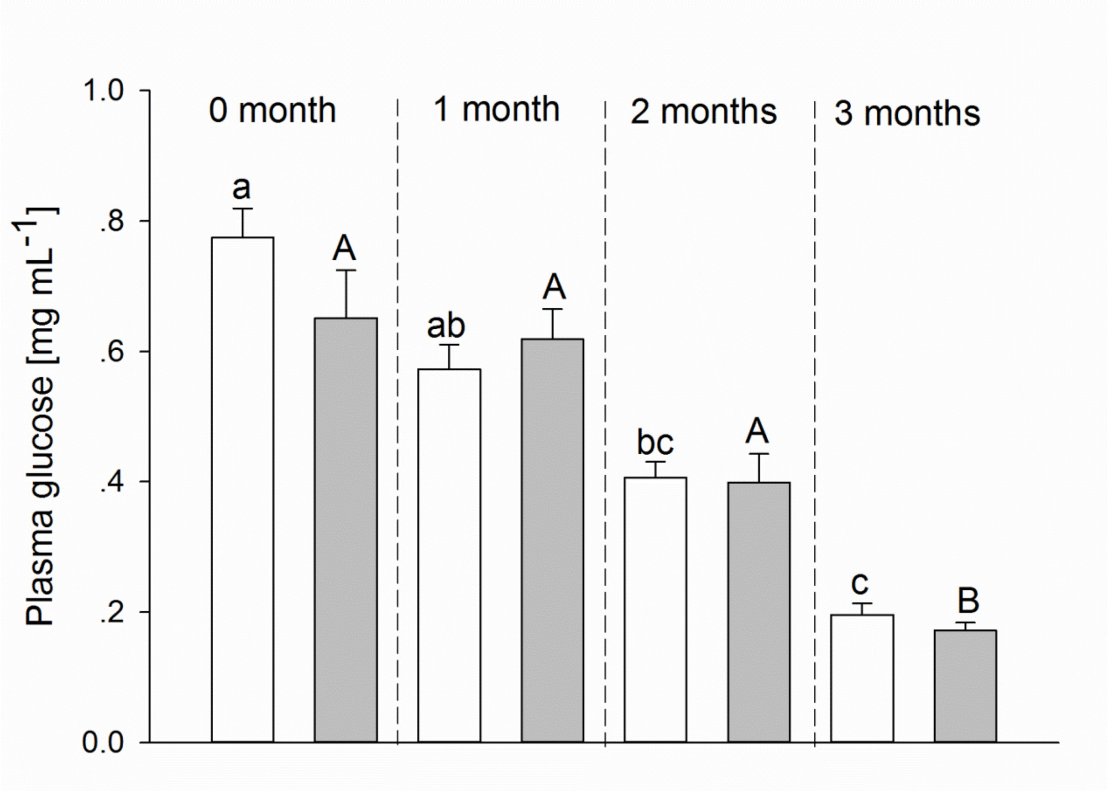

Figure 19 The plasma glucose of the mirror carp receiving transient water stops alone (open bars) or simultaneous 1 mg L^{-1} PAA treatments (grey bars) during different samplings.

The letters a, ab, bc and c indicate homogenous subsets of the control group along time.

The letters A and B indicate homogenous subsets of the treatment group along time.

Error bars indicate the standard error.

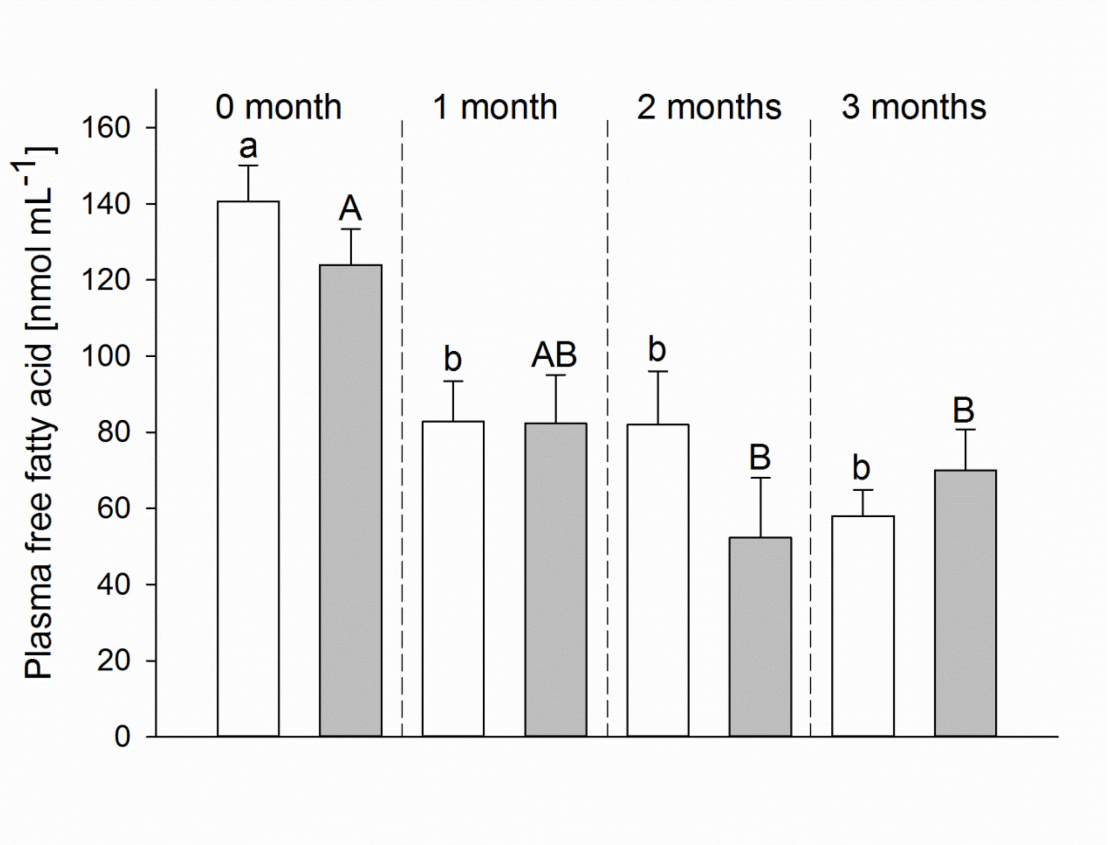

Figure 20 The plasma free fatty acid of the mirror carp receiving transient water stops alone (open bars) or simultaneous 1 mg L^{-1} PAA treatments (grey bars) during different samplings. The letters a and b indicate homogenous subsets of the control group along time. The letters A ,AB and B indicate homogenous subsets of the treatment group along time. Error bars indicate the standard error.

3.2.4. Respiratory burst of kidney leucocytes

The stimulation factor (SF) of PMA on the respiratory burst of head and trunk kidney leucocytes was mostly similar between control and treatment groups. The only exception was during the 3rd sampling, when the mirror carp received treatments for 2 months, the SF of PMA on the respiratory burst of trunk kidney leucocytes of the treatment group was higher than the control group (*df*=10, P=0.007; Figure 21).

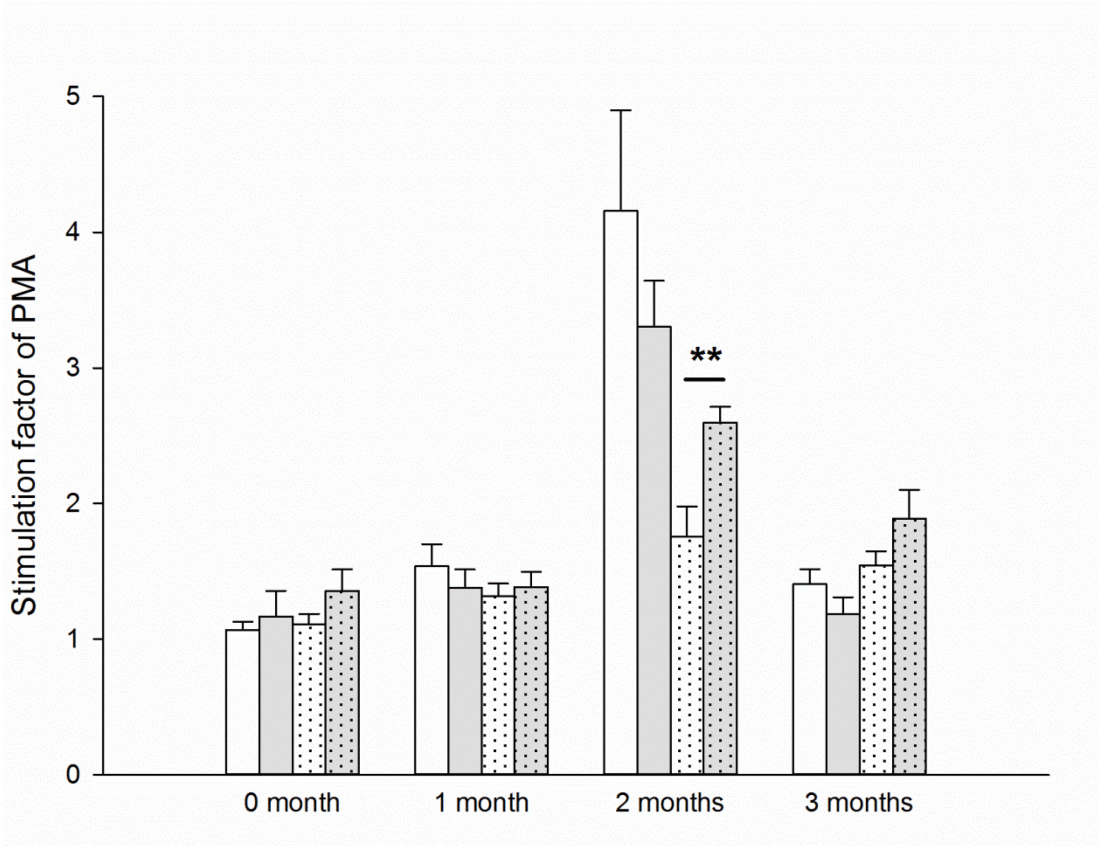

Figure 21 Stimulation factor of PMA on the respiratory burst of head (undotted bars) and trunk (dotted bars) kidney leucocytes of the mirror carp receiving transient water stops alone (white bars) or simultaneous 1 mg L^{-1} PAA treatments (grey bars) during periodic samplings.

**: P<0.01 according to T-test between two groups.

Error bars indicate the standard error.

3.2.5. Gill alteration

The main observed gill alterations in mirror carp were hyperplasia and the aggregation of eosinophilic granulocytes in the interlamellar region (Figure 22). Hyperplasia was equally present in mirror carp from both groups, regardless of the severity (*df*=35, P=0.301; Figure 23). The total quantified gill alteration caused by hyperplasia was similar in mirror carp from

both groups, too (*df*=10, P=0.205; Figure 24). In contrast, aggregation of eosinophilic granulocytes were more frequently present in mirror carp receiving transient water stops alone than those receiving simultaneous PAA treatments (*df*=35, P=0.004; Figure 23). The total quantified gill alteration caused by eosinophilic granulocytes was greater in mirror carp receiving transient water stops alone than those receiving simultaneous PAA treatments (*df*=10, P=0.025; Figure 24).

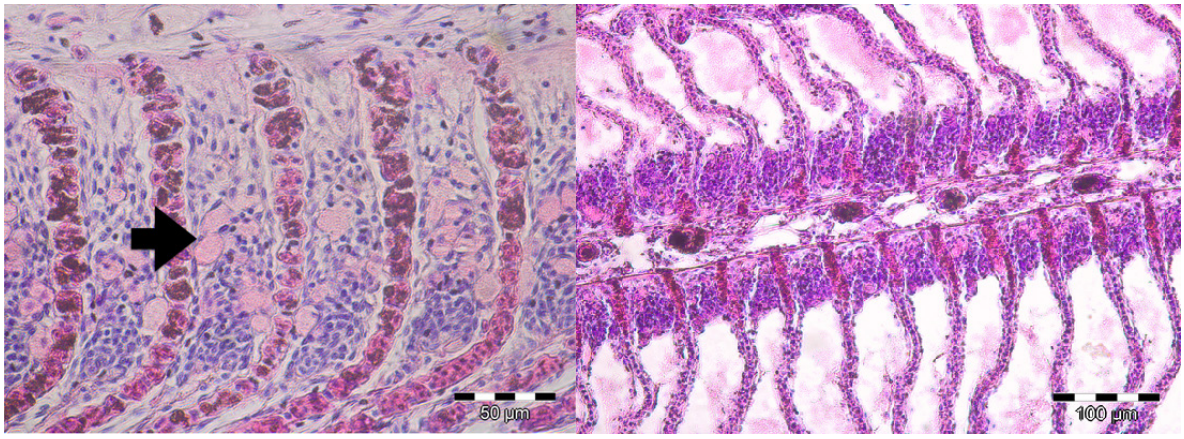

Figure 22 Examples of aggregation of eosinophilic granulocytes (left, marked by the arrow) and hyperplasia in the interlamellar region of mirror carp.

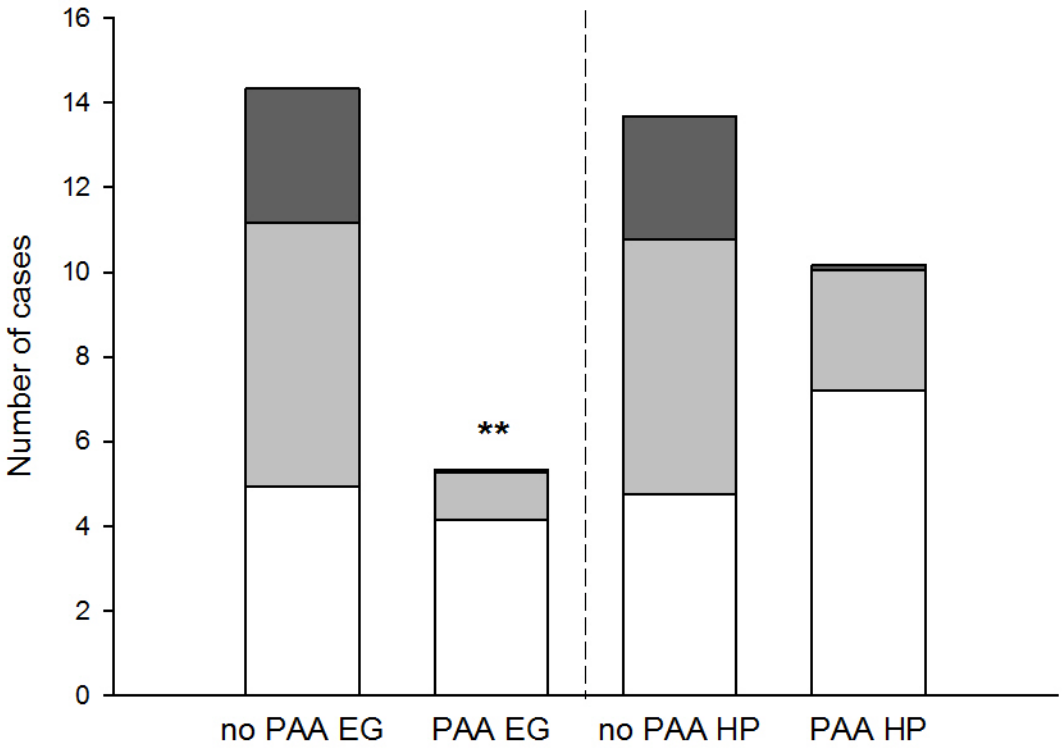

Figure 23 The average presence (number of cases) of eosinophilic granulocytes (EG, left two columns) and hyperplasia (HP, right two columns) divided in severities of 'minimal' (open bars), 'moderate' (grey bars) and 'severe' (black bars) in gill samples of mirror carp receiving transient water stops alone (no PAA) and simultaneous 1 mg L^{-1} PAA treatments (PAA).

**: P<0.01 according to two-way ANOVA analysis.

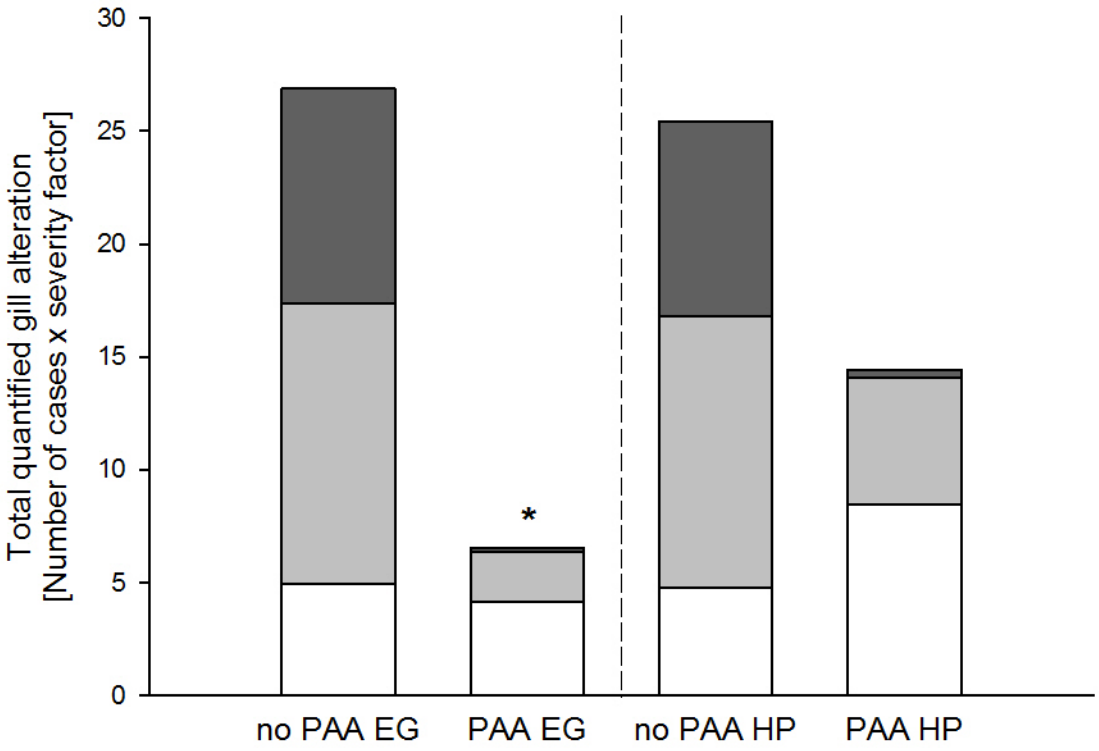

Figure 24 The average total quantified alteration of eosinophilic granulocytes (EG, left two columns) and hyperplasia (HP, right two columns) divided in severities of 'minimal' (open bars), 'moderate' (grey bars) and 'severe' (black bars) in gill samples of mirror carp receiving transient water stops alone (no PAA) and simultaneous 1 mg L^{-1} PAA treatments (PAA).

*: P<0.05 according to T-test.

3.2.6. Bacterial density in the rearing water with or without PAA treatments

Before transient water stops, the total heterotrophic bacterial density (CFU mL^{-1}) in water samples from both groups were similar, regardless of the sampling dates (*df*=11, P=0.054; Figure 25). After transient water stops, the total heterotrophic bacterial density increased up to 6 folds in water samples without PAA treatments, while it decreased for nearly 90% in water samples with simultaneous PAA treatments.

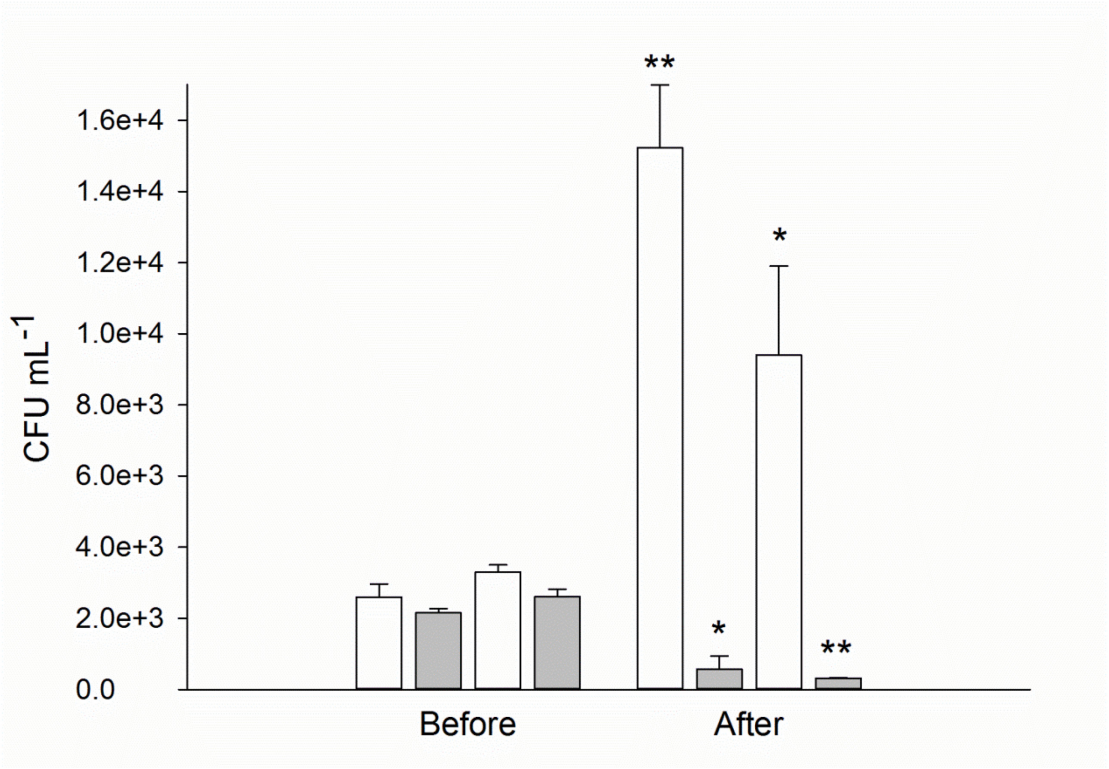

Figure 25 The total heterotrophic bacterial density, demonstrated as colony forming unit (CFU) per mL, in water samples from fish tanks treated with transient water stops alone (open bars) and simultaneous 1 mg L^{-1} PAA treatments (grey bars) before and after transient water stops on two random days.

* and **: P<0.05 and 0.01, respectively, according to the T-test between 'before' and 'after' data.

Error bars indicate the standard error.

4. Discussion

4.1. Stress adaptation of rainbow trout to PAA

Cortisol is predominantly released from the branchial blood vessels of the gill through passive diffusion (Vermeirssen and Scott, 1996). Based on this theory, measurement of water cortisol instead of plasma cortisol has been established and proven to be a reliable indicator of stress for various fish species, especially in flow-through systems (Ruane and Komen, 2003; Ellis et al., 2004; Ellis et al., 2007; Scott and Ellis, 2007; Sebire et al., 2007; Fanouraki et al., 2008; Wong et al., 2008).

In the present study, the pulse treatment of 1 mg L^{-1} PAA visually triggered behavioral responses of rainbow trout indicating stress response, and induced a strong increase of water cortisol in the 1st week. The water cortisol concentration remained higher than the control group until the second day. These facts indicated that rainbow trout were stressed by the first pulse PAA treatment in the 1st week. In contrast, the continuous application led to water cortisol and fish behavior similar to the control group throughout the experiment. It indicated that the continuous PAA treatment did not provoke stress to the rainbow trout at any time. The reason was probably that the actual PAA concentration caused by the continuous application was too low and below a triggering threshold for the rainbow trout to react.

From the 2nd week, the behavioral reaction of rainbow trout to the pulse treatments of PAA was still present but became shorter and less intensive. It indicated rainbow trout could still cognitively sense the pulse treatments. Despite of that, all groups showed similar water cortisol, indicating the pulse PAA treatment caused reduced stress response in rainbow trout

from the 2nd week.

The results indicated a process that pulse treatment of 1 mg L^{-1} PAA induced stress response to rainbow trout during the first exposure. The stress response was downregulated and became insignificant during the successive exposures. Two possible explanations may arise: adaptation or exhaustion. If rainbow trout were exhausted, they were likely to suffer from chronically elevated metabolic activity and subsequently show reduced growth, suppressed immunity and reduced stress response against other stressors (Bonga, 1997; Harris and Bird, 2000; Magnadóttir, 2006). However, in the present study, rainbow trout in all groups showed similar growth. The results of leucocytes respiratory burst indicate unaffected innate cellular immunity. The unchanged activity of antiprotease, esterase, alkaline phosphatase, lysozyme and myeloperoxidase in serum suggests that the innate humoral immunity was unaffected by the pulse PAA treatments. Moreover, rainbow trout receiving pulse PAA treatments showed similar stress response to an additional stressor (dipnet harassment) as the untreated fish. All these facts indicate unaffected fish welfare by the pulse PAA treatments. Therefore, the possibility of exhaustion could be excluded and it can be confirmed that the observed results were due to the adaptation of rainbow trout.

4.2. Non-treatment related variation in the water cortisol concentrations

A few apparently random outliers were encountered in some systems regardless of treatment or time of sampling. They occurred in one fish tank out of triplicates in a random group. Rainbow trout in the outlier tanks showed either aggressive or evading behaviors, while those in the other replicate tanks within the same group were uniformly and peaceful.

That was most likely the consequence of social stress. The dominant fishes can induce acute or chronic stress to the subordinate fishes (Øverli et al., 1999; Sloman et al., 2001; Jeffrey et al., 2014). In the present study, the elevated cortisol value was present rather in short term and unrepeated. Therefore, there was only acute social stress in the outlier tanks. In this case, the subordinate rainbow trout would show prolonged elevated plasma cortisol. As a result, prolonged high water cortisol concentrations tended to be measured in these tanks.

4.3. Oxidative stress induced by peracetic acid at different application strategies

The samples of TAC and ROS/RNS were collected on one day without pulse PAA treatment. Due to the decay of PAA and the flow-through design, no PAA residue was supposed to be present in tanks of the pulse treatment group during the sampling. In contrast, the continuous PAA treatment was constantly present. Despite of the fact that no PAA was measurable in the continuous treatment group, trace amount of PAA was probably still present due to the constant input of PAA stock solution.

Gill is the organ in charge of gas exchange. For this purpose, it has a large surface that is exposed to considerable water flow (Evans et al., 2005). Therefore, gill is theoretically most affected by PAA. The results showed that rainbow trout in the continuous treatment group had higher TAC and ROS/RNS in gill than the control fish. In contrast, rainbow trout in the pulse treatment group had similar TAC and ROS/RNS in gill to the control fish. This difference, firstly, verifies the presence of trace amount of PAA in the continuous treatment group and absence of PAA in the pulse treatments group during the sampling; and secondly, indicates that even trace amount of PAA can induce oxidative stress in gill. Based on this finding, it can

be speculated that the higher PAA concentration during pulse treatments could induce stronger oxidative stress and antioxidative defense in gill. Due to the rather high baseline TAC measured in gill, the increased ROS/RNS is likely to be quickly scavenged. Therefore, the PAA-induced oxidative stress in gill was rather instant than long-term, otherwise an increase of TAC and ROS/RNS in gill should have been measured in rainbow trout receiving pulse PAA treatments, too.

Compared to gill, the TAC and ROS/RNS in serum showed different changes in response to PAA application strategies. Rainbow trout in the pulse treatment group had higher ROS/RNS in serum than the control group, whereas rainbow trout in the continuous treatment group had similar ROS/RNS in serum to the control group. As discussed above, PAA was absent in tanks receiving pulse PAA treatments but present at trace concentration in tanks receiving the continuous PAA treatment during the sampling. Therefore, the increased ROS/RNS in serum of rainbow trout receiving pulse PAA treatments may only be the consequence of either endogenous ROS/RNS production or the residue of diffused PAA. The endogenous ROS/RNS production may be originated from the observed short-term behavioral reaction of rainbow trout in response to pulse PAA treatments. The behavior reaction could be recognized as additional physical exercises, which have been proven to increase the ROS/RNS production in muscle cells. The increased intracellular ROS/RNS could be released to the extracellular liquid and further to the circulating blood (Powers and Jackson, 2008). Rainbow trout in the continuous treatment group, however, had no such behavioral reaction. Therefore, they should have no exercise-induced endogenous ROS/RNS production. Despite of that, the TAC in serum of rainbow trout in the continuous treatment

group was still higher than the control group. This could only be induced by the continuous PAA treatment. PAA products consist of two functional substances: PAA and H_2O_2. Both are oxidizing agents belonging to the family of ROS. Because of low molecular mass, their intermediate degradation products (hydroxyl and hydroperoxyl radicals) or themselves, are likely to be gill-permeable (Wilhelm et al., 1994). The trace amount of PAA during continuous treatment may have diffused from water through the gill into the blood. As exogenous ROS, they further induce the increase of TAC. Because the PAA concentration during continuous treatment was too low, the ROS/RNS in serum was not significantly elevated. In contrast, PAA was present at much higher concentration (0.7 mg L^{-1}) during pulse treatments. Consequently, considerable amount of PAA, as exogenous ROS, was likely to diffuse into the blood. Due to the low baseline TAC in serum, the ROS/RNS-scavenging efficiency was not as high as in the gill. Therefore, residue of diffused PAA was still present in serum several days after the pulse PAA treatment, resulting in a higher serum ROS/RNS concentration than the control group.

The TAC and ROS/RNS in liver of rainbow trout receiving both PAA application strategies showed no difference from the control trout. This was partly due to the antioxidant defense in gill and serum, which decreased the ROS/RNS concentration finally reaching the liver. In addition, the baseline ROS/RNS concentration in liver was higher than in gill and serum. Even after PAA treatments, the measured ROS/RNS concentration in serum did not exceed that in liver. In this case, it is unlikely that ROS/RNS may diffuse to the liver and induce the antioxidant response.

4.4. Impact of peracetic acid on the antiprotease activity in serum

The only affected enzyme in serum of rainbow trout was the antiprotease. The function of antiprotease is that it binds, inactivates protease and prevents tissue destruction. In the process of inflammation, the antiprotease-protease balance is disturbed by the excessive ROS/RNS production, which oxidizes and inactivates antiprotease (Conner and Grisham, 1996). According to this theory, the antiprotease activity in serum of rainbow trout should have be reduced by the pulse PAA treatments instead of continuous PAA treatment, because the ROS/RNS increase was measured in serum of rainbow trout receiving pulse PAA treatments instead of the continuous PAA treatment. However, the measured antiprotease activity in serum of rainbow trout was significantly reduced by the continuous PAA treatment for about 70%, compared to the control group. In contrast, the pulse PAA treatments caused an insignificant reduction of antiprotease activity in serum, which was similar to that caused by the continuous PAA treatment. This result is not necessarily contrary to the theory. In case of continuous PAA treatment, although it induced minor oxidative stress in serum, the oxidative stress was rather statically present due to the constant input of PAA stock solution. In case of pulse PAA treatments, although they induced stronger oxidative stress in serum, the oxidative stress could be dynamically neutralized (recovered) during the treatment intervals. During the recovery phase after pulse PAA treatments, the excessive ROS in serum was decreasing, resulting in a reduced inactivating effect on the antiprotease and further a recovery of its activity. This process, however, was likely absent in rainbow trout receiving the continuous PAA treatment.

As discussed above, the antiprotease activity in serum of rainbow trout was likely to

undergo a periodic reduction-and-recovery process after each pulse PAA treatment, versus a static reduction after the continuous PAA treatment. A static reduction of serum antiprotease activity is often observed in many chronic inflammatory diseases (Streit et al., 1995; Cox, 2009; Parameswaran et al., 2009; Meyer et al., 2014). Therefore, there is a risk that the continuous PAA treatment might induce chronic inflammation in fish. To verify this risk, further evidence is needed in future studies.

4.5. Gill alteration caused by peracetic acid in optimal water quality

In general, the only observed gill alteration of rainbow trout caused by peracetic acid treatments was hyperplasia, and mostly in 'minimal' severity. Visually, there was no obvious difference among the gill slides of rainbow trout from different groups. Statistically, the pulse applications of PAA did cause a higher presence of 'minimal' hyperplasia than the untreated control. However, both pulse and continuous PAA applications resulted in a similar presence of 'minimal' hyperplasia. Moreover, the presence of 'moderate' hyperplasia, as well as the total quantified gill alteration, was identical in all groups. Therefore, the impact of the high-dose PAA during pulse applications on the gill histology was too minimal to distinguish from the low-dose PAA during continuous application. Noteworthy, the 'low-dose' PAA during continuous application in the present study was lower than the detectable range of the described DPD-method. Therefore, the observed effect was based on the continuous input of trace amount of PAA. If a true concentration of 0.2 mg L^{-1} PAA was maintained during continuous application, its negative impact on gill histology is predicted to be greater.

4.6. Impact of PAA applications on biofilm formation and water quality

Oxygen, pH and nitrogenous compounds (ammonium, nitrite and nitrate) are important indicators for water quality in aquaculture. Their variations are usually the combined consequence of water retention, stocking density, feed input and microbial activities. In the present study, systems in all groups had identical water retention, stocking density and feed input. Therefore, the difference of water quality among groups was mainly due to the difference on microbial activities, which, in aquaculture, are present in planktonic phase and in the biofilm. The biofilm, with similarities to periphyton, is a ubiquitous organic matrix consisting of extra polymeric substances, bacteria and periphytic algae that coevolves with their planktonic cells during conditions with nutrient load (van Dam et al., 2002; Rao, 2010). In the present study having tanks with a relatively short retention time of 9 hours, the planktonic cells were constantly flushed away by the flow-through design. Consequently, the microbial activities in planktonic phase were less pronounced, compared to those in the biofilm. Especially in RAS, the attached biofilm has been extensively used for the design of the nitrifying biofilter (Chen et al., 2006).

The pulse treatments of 1 mg L^{-1} PAA nearly completely removed the biofilm in the fish tank with the given exposure time. The biofilm formation begins with accumulation of organic molecules on a surface followed by bacterial colonization (Wahl, 1989). As PAA was applied shortly after fish were stocked in the systems, when the biofilm was in early development stage, the pulse PAA treatments probably inhibited the early bacterial colonization based on the reported general antibacterial effect (Kitis, 2004). Surprisingly, microbial nitrification was not completely but partly inhibited, which is in line with previous

finding (Pedersen et al., 2009). Nitrification still occurred in week 5, seen as elevated but slightly lower nitrate-N levels compared to the other two treatment groups. The nitrifying bacteria, as reported by Chandran and Love (2008), showed tolerance to the batch treatment of 0.1 mM H_2O_2 with recovered nitrification activity. In the present study, a comparable concentration of PAA was used, which probably led to a similar minor inhibitory effect on nitrifying bacteria. Compared to autotrophic nitrifying bacteria, the heterotrophic bacteria, especially the aerobics, seemed to be strongly inhibited by the pulse PAA treatments. The inhibitory effect could be interpreted by the highest daily oxygen concentration measured in the pulse treatment group, which indicates the lowest microbial oxygen consumption. Moreover, the pulse PAA treatments resulted in a stable pH of 7.7 - 7.8 within the first 4 weeks. This suggests that the CO_2 production caused by microbial aerobic respiration was minimal and stable. The competition of heterotrophic bacteria and autotrophic nitrifying bacteria in biofilms, as reported by Wik and Breitholtz (1996), is affected by various parameters. In the present study, the nitrifying bacteria probably dominated the biofilm due to high susceptibility to PAA. This further supports the observed results that the nitrification was not completely inhibited by the pulse PAA treatments.

Despite of higher weekly PAA input than the pulse PAA treatment (360 mg PAA per week), the continuous PAA treatment (672 mg PAA per week) did not cause PAA accumulation and the resulting concentration was close to or below detection limit. This was probably caused by fast degradation, which was potentially attenuated by microbial adaptation. Acetic acid and acetate are active ingredients in PAA trade products. Both potentially contribute to microbial growth as well-known easy degradable dissolved organic

matter. The addition of organic carbon may enhance the growth of periphytic algae and heterotrophic bacteria in the biofilm (Asaduzzaman et al., 2008). This was visually confirmed by the enhanced biofilm formation in tanks receiving continuous PAA treatment, compared to the control group. Indications of biofouling on the pH probes in the control and continuous treatment groups was also observed, but the corresponding pH changes in each treatment group diverged. The pH of the control group decreased, while that of the continuous PAA treatment group slightly increased. This suggests a different composition of microbes in the biofilm. Although a diagnosis of the biofilm was not performed, based on the pH increase solely, it could be suggested that phototrophic/autotrophic algae were potentially favored over bacteria. This, in addition, could explain that the oxygen concentration measured in the continuous treatment group was higher than the untreated control group. The underlying mechanism was probably that a continuous input of trace amount of PAA, as exogenous ROS, might be still enough to induce oxidative stress to microorganisms. Bacteria, especially in growing phase, have low tolerance to the oxidative stress (Sigler et al., 1999). For survival under oxidative stress, bacteria must increase the synthesis of antioxidant enzymes, which consumes extra energy and thus inhibits their reproduction. In contrast, algae undergo photoprotective processes, which protect them from oxidative damage and increase their tolerance to oxidative stress (Niyogi, 1999). As a consequence, the continuous PAA treatment was a selective pressure to microbial composition in the biofilm, where the periphytic algae and autotrophic nitrifying bacteria were favored over heterotrophic bacteria. Despite of enhanced biofilm formation, the continuous PAA treatment did not result in enhanced nitrification. Instead, similar

concentrations of TAN, nitrite-N and nitrate-N were measured in the control and continuous treatment group. The bacterial nitrification is affected by the stratified diffusion of TAN and O_2 in the biofilm. The concentrations of TAN and O_2 decrease from the surface of the biofilm to the attached medium (Chen et al., 2006). As a result, the bacterial nitrification was mostly active in the surface area of the biofilm, whereas limited in the subsurface area. Moreover, the flow-through design continuously flushed the TAN away, resulting in a TAN-limited condition. In this case, the bacterial nitrification of the control and continuous PAA treatment groups was limited by the available TAN. Therefore, a difference of nitrite/nitrate was unlikely to be present.

4.7. Pulse versus continuous application strategies

The differences between the pulse and continuous application strategies were the applied PAA concentration and the duration. The pulse applications used in a higher PAA concentration with shorter duration. They resulted in a minimal but adaptive stress in fish, minimal hyperplasia in gill, as well as partly inhibited nitrification. Despite of that, the growth and immunity of rainbow trout were unaffected. Although the pulse PAA applications induced oxidative stress, the periodic recoveries during application intervals lowered the potential risk of inflammation. Moreover, the highest oxygen concentration and a stable pH as results of pulse PAA applications insured a long-term good living condition for fish. Despite of strong antimicrobial effect, the impact of pulse PAA applications on nitrification can be minimized by bypassing the biofilter or dilution effect of the system hydraulics. In contrast, the continuous application of PAA was supposed to maintain a PAA

concentration of 0.2 mg L^{-1} in the water. This was, unfortunately, not realized in the present study. The reason was apparently a faster decay of PAA than expected due to fish and microbial activities. The realized PAA concentration was too low to stress the fish, as well as to affect their growth and immunity. Despite of the too low concentration, it induced a mild oxidative stress in fish, which was constantly present and induced a reduction of antiprotease activity in serum. A long-term reduction of antiprotease activity may result in chronic inflammation in fish. Moreover, a mild antimicrobial effect of the continuous PAA application was observed, whereas the nitrification was unaffected. Despite of that, enhanced biofilm formation was observed. The enhanced biofilm formation may result in 1) the blockage of the outflow and extra labor for dredge; 2) the increase of opportunistic pathogens under the protective shed of the biofilm. Taking all results in consideration, the biweekly pulse PAA applications at 1 mg L^{-1} are superior to the continuous PAA application at 0.2 mg L^{-1}.

4.8. Beneficial effect of PAA applications on the fish health in bad water quality

The baseline plasma cortisol concentration of adult cyprinids, as documented in literatures, is normally under 40 ng mL^{-1} (Ruane et al., 2002b; Ruane et al., 2002a). After the 1st transient water stop, the average plasma cortisol concentration of mirror carp was elevated up to about 550 ng mL^{-1}. This indicates that the mirror carp was stressed by the 1st transient water stop. Subsequently, the stress response induced by transient water stops was progressively downregulated, as indicated by the reducing plasma cortisol concentration along samplings. During the last sampling, when mirror carp had received periodic transient

water stops for 3 months; their average plasma cortisol concentration was as low as 7 ng mL^{-1}.

The simultaneous 1 mg L^{-1} PAA treatments accompanied with transient water stops showed a stress reduction effect during the first 2 samplings. This was indicated by the results that mirror carp receiving simultaneous PAA treatments showed significant lower plasma cortisol concentration than those receiving transient water stops alone. Afterwards, the stress reduction effect of 1 mg L^{-1} PAA treatments discontinued, because mirror carp receiving simultaneous PAA treatments showed similar or higher plasma cortisol concentration than those receiving transient water stops alone. This fact indicates that the 1 mg L^{-1} PAA treatment alone was a stressor to mirror carp, too. Moreover, a stress downregulation was also observed in mirror carp receiving simultaneous PAA treatments.

Despite of low feeding rate and feed withdrawing, the transient water stops alone caused a 6-fold increase of heterotrophic bacterial density in the rearing water. As a result, the water quality dramatically dropped and induced stress in mirror carp. In contrast, simultaneous PAA treatments resulted in 90% decrease of heterotrophic bacterial density, which avoided the deterioration of the water quality. Therefore, the mechanism that the PAA treatments modified the stress response of carp was probably that they may have overwritten the stress induced by the transient water stops alone. The plasma glucose and FFA was decreasing along samplings, this was probably due to the observed gonadal development. Despite of that, the plasma glucose and FFA concentrations were constantly similar in mirror carp, regardless of the simultaneous PAA treatments. This indicates that the stress response of the mirror carp induced by either transient water stops or PAA treatments

was rather acute that the energy allocation was unaffected. Because both water stops and simultaneous PAA treatments were rather short-term and periodic, the mirror carp from both groups were, in most time, stocked at the same water condition. This could be supported by the same bacterial density measured in tanks of both groups before transient water stops.

The 1 mg L^{-1} PAA treatments resulted in a better gill condition of the mirror carp. The improved gill health was mainly represented by less intensive aggregation of eosinophilic granulocytes in the interlamellar region. The eosinophilic granulocytes aggregated in tissues are migrated from the hematopoietic system through the blood circulation (Powell et al., 1990). Their presence in fish tissues is associated with the immunological defense and inflammatory reactions (Reite and Evensen, 2006). Especially in gill, bacterial infection can result in an increased aggregation of eosinophilic granulocytes (Fla ÑO et al., 1996). In the present study, the transient water stops strongly increased the bacterial density in the rearing water, while the simultaneous PAA treatments strongly decreased the bacterial density. Exposure to higher bacterial density was likely to trigger the increased aggregation of eosinophilic granulocytes in gill.

In most time, similar hematocrits and respiratory burst of kidney leucocytes were measured in mirror carp receiving transient water stops alone and simultaneous 1 mg L^{-1} PAA treatments. The only exception was that during the 3rd sampling, mirror carp receiving transient water stops alone showed a lower hematocrit and a weaker stimulating effect of PMA on the respiratory burst of trunk kidney leucocytes. The decrease of hematocrit was often observed after infections in cyprinids (Yildiz, 1998; Harikrishnan et al., 2003; Ahmed et

al., 2011) , as well as in other fish species (Foda, 1973; Peña-Rehbein et al., 2013; Jerônimo et al., 2014). In the present study, the decrease of hematocrit was apparently not caused by changes of plasma osmolality. Therefore, infection was likely to be present in mirror carp receiving transient water stops alone. This infection was probably cause by the increasing bacterial density during water stops, while prevented by the simultaneous PAA treatments. The responsible pathogens for this infection were likely to be the opportunistic pathogenic bacteria, such as *Aeromonas*, *Flavobacterium*, and *Vibrio*, that are ubiquitous in aquaculture systems (Derome et al., 2016). Moreover, this infection seemed to result in the invasion of pathogenic bacteria in the trunk kidney. Consequently, the leucocytes in trunk kidney had been already stimulated by the bacterial infection before the 3rd sampling. Therefore, a weaker stimulating effect of PMA on the respiratory burst of trunk kidney leucocyte was observed. The signs of infection, however, disappeared during the 4th sampling, indicating a recovery of mirror carp from infection. The recovery was likely to be favored by many factors: 1) the immunologic defense from the fish; 2) the down-regulated stress response in fish; 3) the reduced stocking density due to samplings and the resulting decrease of feed input in the system.

The kidney leucocytes of mirror carp had stronger respiratory burst during the 3rd sampling than the other samplings. This was probably due to incubation of kidney fragments in collagenase-supplemented wash medium prior to isolation. The mechanical isolation was found to cause the highest degree of cell damage, while the incubation in collagenase-supplemented medium was found to cause the least cell damage and preserve the highest cell viability (Jung et al., 1995). Therefore, it is recommended to use collagenase

incubation for the kidney leucocytes isolation in future studies.

5. Conclusion and perspectives of future research

In optimal water quality, PAA can stress naïve fish. There seems to be a threshold concentration of PAA to trigger the stress response of fish. The commonly used concentration, 1 mg PAA L^{-1}, is beyond of this threshold, while under 0.2 mg PAA L^{-1} is probably below it. In this aspect, future research could seek the PAA threshold and the mechanism of how fish sense PAA.

The stress response induced by 1 mg L^{-1} PAA is adaptive for fish. They can down-regulate the cortisol release, and show unaffected growth and innate immunity. Moreover, PAA can induce slight hyperplasia in fish gill, depending on the applied concentration. Despite of that, the overall gill health is unlikely to be affected.

PAA, as exogenous ROS, is probably gill-permeable. Even at trace concentrations, PAA can induce oxidative stress in gill and serum. Higher PAA concentration is likely to induce stronger oxidative stress and antioxidative defense in fish. The periodic exposures to 1 mg L^{-1} PAA result in periodic up- and down-regulation of the antioxidative defense in fish. In contrast, the continuous exposure to trace amount of PAA results in constant up-regulation of the antioxidative defense. The latter is more risky due to a potential induction of chronic inflammation. To verify this, future research could measure several inflammatory markers in different organs of fish exposed to different PAA treatment strategies.

PAA has a concentration-dependent antimicrobial effect. Various microbes show differing susceptibility to PAA-induced oxidative stress. Biweekly applications of 1 mg L^{-1} PAA can strongly inhibit the biofilm formation and microbial activities. In addition, the nitrification can be partly inhibited, too. In contrast, the continuous application of 0.2 mg L^{-1}

PAA is likely to have a fast PAA decay. The continuous application of low concentration of PAA can enhance the biofilm formation. Moreover, it has a slight antimicrobial effect against heterotrophic bacteria, while the autotrophic nitrifying bacteria are likely unaffected.

In production-scale aquaculture facilities, the optimal water quality is rare. Instead, high organic load and microbial density in the rearing water is common. Many failures can even worsen the water quality and induce extra stress to fish. Consequently, a high risk of infections is often present. In this case, regular applications of PAA at 1 mg L^{-1} can effectively improve the water quality and overwrite the stress induced by the deterioration of water quality. In addition, the gill health can be improved, infections can be prevented and the overall fish health can be enhanced.

Based on effective antimicrobial performance, ease of use and low risks to affect fish health, PAA is recommended to be applied periodically at rather high concentrations (1-2 mg L^{-1}) with sufficient intervals. In production-scale aquaculture facilities, regular applications of PAA are especially beneficial to fish health and should be set as a routine hygiene measure.

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe. Des Weiteren erkläre ich meine Kenntnisnahme der dem angestrebten Verfahren zugrunde liegenden Promotionsordnung. Ich habe mich anderweitig nicht um einen Doktorgrad beworben und bin nicht im Besitz eines entsprechenden Doktorgrads.

Berlin, den 24.11.2016

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